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(54) Title: CAUSATIVE AGENT OF THE MYSTERY SWINE DISEASE, VACCINE COMPOSITIONS AND DIAGNOSTIC KITS

(57) Abstract

Composition of matter comprising the causative agent of Mystery Swine Disease, Lelystad Agent, in a live, attenuated, dead, or recombinant form, or a part or component of it. Vaccine compositions and diagnostic kits based thereon. Recombinant nucleic acid comprising a Lelystad Agent-specific nucleotide sequence. Peptides comprising a Lelystad Agent-specific amino acid sequence. Lelystad Agent-specific antibodies.

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Title: Causative agent of the Mystery Swine Disease, vaccine compositions and diagnostic kits

FIELD OF THE INVENTION

The invention relates to the isolation, characterization and utilization of the causative agent of the Mystery Swine Disease (MSD). The invention utilizes the discovery of the agent causing the disease and the determination of its genome organization, the genomic nucleotide sequence and the proteins encoded by the genome, for providing protection against and diagnosis of infections, in particular protection against and diagnosis of MSD infections, and for providing vaccine compositions and diagnostic kits, either for use with MSD or with other pathogen-caused diseases.

BACKGROUND

In the winter and early spring of 1991, the Dutch pig industry was struck by a sudden outbreak of a new disease among breeding sows. Most sows showed anorexia, some aborted late in gestation (around day 110), showed stillbirths or gave birth to mummified fetuses and some had fever. Occasionally, sows with bluish ears were found, therefore the disease was commonly named "Abortus Blauw". The disease in the sows was often accompanied by respiratory distress and death of their young piglets, and often by respiratory disease and growth retardation of older piglets and fattening pigs.

The cause of this epizootic was not known, but the symptoms resembled those of a similar disease occurring in Germany since late 1990, and resembled those of the so-called "Mystery Swine Disease" as seen since 1987 in the mid-west of the United States of America and in Canada (Hill, 1990). Various other names have been used for the disease, in Germany it is known as "Seuchenhafter Spätabort der Schweine", and in North-America it is also known as "Mystery Pig Disease", "Mysterious Reproductive Syndrome", and "Swine Infertility and Respiratory Syndrome". In North-America, Loula (1990) described the general clinical signs as:

1) Off feed, sick animals of all ages
2) Abortions, stillbirths, weak pigs, mummies
3) Post farrowing respiratory problems
4) Breeding problems.

No causative agent has as yet been identified, but encephalomyocarditis virus (EMCV), porcine parvo virus (PPV), pseudorabies virus (PRV), swine influenza virus (SIV), bovine viral diarrhea virus (BVDV), hog cholera virus (HCV), porcine entero viruses (PEV), an influenza-like virus, chlamidiae, leptospirae, have all been named as possible cause (Loula, 1990; Mengeling and Lager, 1990; among others).

SUMMARY OF THE INVENTION

The invention provides a composition of matter comprising isolated Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102. The words "essentially corresponding" refer to variations that occur in nature and to artificial variations of Lelystad Agent, particularly those which still allow detection by techniques like hybridization, PCR and ELISA, using Lelystad Agent-specific materials, such as Lelystad Agent-specific DNA or antibodies.

The composition of matter may comprise live, killed, or attenuated isolated Lelystad Agent; a recombinant vector derived from Lelystad Agent; an isolated part or component of Lelystad Agent; isolated or synthetic protein, (poly)peptide, or nucleic acid derived from Lelystad Agent; recombinant nucleic acid which comprises a nucleotide sequence derived from the genome of Lelystad Agent; a (poly)peptide having an amino acid sequence derived from a protein of Lelystad Agent, the (poly)peptide being produced by a cell capable of producing it due to genetic engineering with appropriate recombinant DNA; an isolated or synthetic antibody which specifically recognizes a part or component of Lelystad Agent;

or a recombinant vector which contains nucleic acid comprising a nucleotide sequence coding for a protein or antigenic peptide derived from Lelystad Agent.

On the DNA level, the invention specifically provides a recombinant nucleic acid, more specifically recombinant DNA, which comprises a Lelystad Agent-specific nucleotide sequence shown in figure 1. Preferably, said Lelystad Agent-specific nucleotide sequence is selected from anyone of the ORFs (Open Reading Frames) shown in figure 1.

On the peptide/protein level, the invention specifically provides a peptide comprising a Lelystad Agent-specific amino acid sequence shown in figure 1.

The invention further provides a vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising Lelystad Agent, either live, killed, or attenuated; or a recombinant vector which contains nucleic acid comprising a nucleotide sequence coding for a protein or antigenic peptide derived from Lelystad Agent; an antigenic part or component of Lelystad Agent; a protein or antigenic polypeptide derived from, or a peptide mimicking an antigenic component of, Lelystad Agent; and a suitable carrier or adjuvant.

The invention also provides a vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against a disease caused by a pathogen, comprising a recombinant vector derived from Lelystad Agent, the nucleic acid of the recombinant vector comprising a nucleotide sequence coding for a protein or antigenic peptide derived from the pathogen, and a suitable carrier or adjuvant.

The invention further provides a diagnostic kit for detecting nucleic acid from Lelystad Agent in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine,

comprising a nucleic acid probe or primer which comprises a nucleotide sequence derived from the genome of Lelystad Agent, and suitable detection means of a nucleic acid detection assay.

The invention also provides a diagnostic kit for detecting antigen from Lelystad Agent in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising an antibody which specifically recognizes a part or component of Lelystad Agent, and suitable detection means of an antigen detection assay.

The invention also provides a diagnostic kit for detecting an antibody which specifically recognizes Lelystad Agent in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising Lelystad Agent; an antigenic part or component of Lelystad Agent; a protein or antigenic polypeptide derived from Lelystad Agent; or a peptide mimicking an antigenic component of Lelystad Agent; and suitable detection means of an antibody detection assay.

The invention also relates to a process for diagnosing whether an animal, in particular a mammal, more in particular a pig or swine, is contaminated with the causative agent of Mystery Swine Disease, comprising preparing a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from the animal, and examining whether it contains Lelystad Agent nucleic acid, Lelystad Agent antigen, or antibody specifically recognizing Lelystad Agent, said Lelystad Agent being the causative agent of Mystery Swine Disease and essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

DETAILED DESCRIPTION OF THE INVENTION

The invention is a result of combined efforts of the Central Veterinary Institute (CVI) and the Regional Animal Health Services (RAHS) in the Netherlands in trying to find the cause of the new disease MSD. Farms with pigs affected by the new disease were visited by field veterinarians of the RAHS. Sick pigs, specimens of sick pigs, and sow sera taken at the time of the acute and convalescent phase of the disease were sent for virus isolation to the RAHS and the CVI. Paired sera of affected sows were tested for antibodies against ten known pig-viruses. Three different viruses, encephalomyocarditis virus, porcine entero virus type 2, porcine entero virus type 7, and an unknown agent, Lelystad agent (LA), were isolated. Sows which had reportedly been struck with the disease mainly seroconverted to LA, and hardly to any of the other virus isolates or the known viral pathogens. In order to reproduce MSD experimentally, eight pregnant sows were inoculated intranasally with LA at day 84 of gestation. One sow gave birth to seven dead and four live but very weak piglets at day 109 of gestation; the four live piglets died one day after birth. Another sow gave birth at day 116 to three mummified fetuses, six dead piglets and three live piglets; two of the live piglets died within one day. A third sow gave birth at day 117 to two mummified fetuses, eight dead and seven live piglets. The other sows farrowed around day 115 and had less severe reproductive losses. The mean number of live piglets from all eight sows at birth was 7.3 and the mean number of dead piglets at birth was 4.6. Antibodies directed against LA were detected in 10 out of 42 serum samples collected before the pigs had sucked. LA was isolated from three piglets that died shortly after birth. These results justify the conclusion that LA is the causal agent of mystery swine disease.

LA grows with a cytopathic effect in pig lung macrophages and can be identified by staining in an immuno-peroxidase-monolayer assay (IPMA) with postinfection sera of pigs c 829

and b 822, or with any of the other postinfection sera of the SPF pigs listed in table 5. Antibodies to LA can be identified by indirect staining procedures in IPMA. LA did not grow in any other cell system tested. LA was not neutralized by homologous sera, or by sera directed against a set of known viruses (Table 3). LA did not haemagglutinate with the red blood cells tested. LA is smaller then 200 nm since it passes through a filtre with pores of this size. LA is sensitive to chloroform. The above results show that Lelystad agent is not yet identified as belonging to a certain virus group or other microbiological species. It has been deposited 5 June 1991 under number I-1102 at Institute Pasteur, France.

The genome organization, nucleotide sequences, and polypeptides derived therefrom, of LA have now been found. These data together with those of others (see below) justify classification of LA (hereafter also called Lelystad Virus or LV) as a member of a new virus family, the Arteriviridae. As prototype virus of this new family we propose Equine Arteritis Virus (EAV), the first member of the new family of which data regarding the replication strategy of the genome and genome organization became available (de Vries et al., 1990, and references therein). On the basis of a comparison of our sequence data with those available for Lactate Dehydrogenase-Elevating Virus (LDV; Godeny et al., 1990), we propose that LDV is also a member of the Arteriviridae.

Given the genome organization and translation strategy of Arteriviridae it seems appropriate to place this new virus family into the superfamily of coronaviruses (Snijder et al., 1990a).

Arteriviruses have in common that their primary target cells in respective hosts are macrophages. Replication of LDV has been shown to be restricted to macrophages in its host, the mouse, whereas this strict propensity for macrophages has not been resolved yet for EAV, and LV.

Arteriviruses are spherical enveloped particles having a diameter of 45-60 nm and containing an icosahedral

nucleocapsid (Brinton-Darnell and Plagemann, 1975; Horzinek et al., 1971; Hyllseth, 1973).

The genome of Arteriviridae consists of a positive stranded polyadenylated RNA molecule with a size of about 12-13 kilobases (kb) (Brinton-Darnell and Plageman, 1975; van der Zeijst et al., 1975). EAV replicates via a 3' nested set of six subgenomic mRNAs, ranging in size from 0.8 to 3.6 kb, which are composed of a leader sequence, derived from the 5' end of the genomic RNA, which is joined to the 3' terminal body sequences (de Vries et al., 1990).

Here we show that the genome organization and replication strategy of LV is similar to that of EAV, coronaviruses and toroviruses, whereas the genome sizes of the latter viruses are completely different from those of LV and EAV.

The genome of LV consists of a genomic RNA molecule of about 14.5 to 15.5 kb in length (estimated on a neutral agarose gel), which replicates via a 3' nested set of subgenomic RNAs. The subgenomic RNAs consist of a leader sequence, the length of which is yet unknown, which is derived from the 5' end of the genomic RNA and which is fused to the body sequences derived from the 3' end of the genomic RNA (Fig. 2).

The nucleotide sequence of the genomic RNA of LV was determined from overlapping cDNA clones. A consecutive sequence of 15,088 bp was obtained covering nearly the complete genome of LV (Fig. 1). In this sequence 8 open reading frames (ORFs) were identified: ORF 1A, ORF 1B, and ORFs 2 to 7.

ORF 1A and ORF 1B are predicted to encode the viral replicase or polymerase, whereas ORFs 2 to 6 are predicted to encode structural viral membrane (envelope) associated proteins. ORF 7 is predicted to encode the structural viral nucleocapsid protein.

Because the products of ORF 6 and ORF 7 of LV show a significant similarity with VpX and Vp1 of LDV respectively,

it is predicted that the sequences of ORFs 6 and 7 will also be highly conserved among antigenic variants of LV.

The complete nucleotide sequence of figure 1 and all the sequences and protein products encoded by ORFs 1 to 7 and possible other ORFs located in the sequence of figure 1, are especially suited for vaccine development, in whatever sense, and for the development of diagnostic tools, in whatever sense. All possible modes are well known to persons skilled in the art.

Since it is now possible to unambigously identify LA, the causal agent of MSD, it can now be tested whether pigs are infected with LA or not. Such diagnostic tests have until now not been available.

The test can be performed by virus isolation in macrophages, or other cell culture systems in which LA might grow, and staining the infected cultures with antibodies directed against LA (such as postinfection sera c 829 or b 822), but it is also feasible to develop and employ other types of diagnostic tests.

For instance, it is possible to use direct or indirect immunohistological staining techniques, i.e. with antibodies directed to LA that are labeled with fluorescent compounds such as isothiocyanate, or labeled with enzymes such as horseradish peroxidase. These techniques can be used to detect LA antigen in tissue sections or other samples from pigs suspected to have MSD. The antibodies needed for these tests can be c 829 or b 822 or other polyclonal antibodies directed against LA, but monoclonal antibodies directed against LA can also be used.

Furthermore, since the nature and organization of the genome of LA and the nucleotide sequence of this genome have been determined, LA specific nucleotide sequences can be identified and used to develop oligonucleotide sequences that can be used as probes or primers in diagnostic techniques such as hybridization, polymerase chain reaction, or any other

techniques that are developed to specifically detect nucleotide acid sequences.

It is also possible to test for antibodies directed against LA. Table 5 shows that experimentally infected pigs rapidly develop antibodies against LA, and table 4 shows that pigs in the field also have strong antibody responses against LA. Thus it can now also be determined whether pigs have been infected with LA in the past. Such testing is of utmost importance in determining whether pigs or pig herds or pig populations or pigs in whole regions or countries are free of LA. The test can be done by using the IPMA as described, but it is also feasible to develop and employ other types of diagnostic tests for the detection of antibodies directed against LA.

LA specific proteins, polypeptides, and peptides, or peptide sequences mimicking antigenic components of LA, can be used in such tests. Such proteins can be derived from the LA itself, but it is also possible to make such proteins by recombinant DNA or peptide synthesis techniques. These tests can use specific polyclonal and/or monoclonal antibodies directed against LA or specific components of LA, and/or use cell systems infected with LA or cell systems expressing LA antigen. The antibodies can be used, for example, as a means for immobilizing the LA antigen (a solid surface is coated with the antibody whereafter the LA antigen is bound by the antibody) which leads to a higher specificity of the test, or can be used in a competitive assay (labeled antibody and unknown antibody in the sample compete for available LA antigen).

Furthermore, the above described diagnostic possibilities can be applied to test whether other animals, such as mammals, birds, insects or fish, or plants, or other living creatures, can be, or are, or have been infected with LA or related agents.

Since LA has now been identified as the causal agent of MSD, it is possible to make a vaccine to protect pigs against

this disease. Such a vaccine can simply be made by growing LA in pig lung macrophage cultures, or in other cell systems in which LA grows. LA can then be purified or not, and killed by established techniques, such as inactivation with formaline or ultra-violet light. The inactivated LA can then be combined with adjuvantia, such as Freund's adjuvans or aluminum hydroxide or others, and this composition can then be injected in pigs.

Dead vaccines can also be made with LA protein preparations derived from LA infected cultures, or derived from cell systems expressing specifically LA protein through DNA recombinant techniques. Such subunits of LA would then be treated as above, and this would result in a subunit vaccine.

Vaccines using even smaller components of LA, such as polypeptides, peptides, or peptides mimicking antigenic components of LA are also feasible for use as dead vaccine.

Dead vaccines against MSD can also be made by recombinant DNA techniques through which the genome of LA, or parts thereof, is incorporated in vector systems such as vaccinia virus, herpesvirus, pseudorabies virus, adeno virus, baculo virus or other suitable vector systems that can so express LA antigen in appropriate cells systems. LA antigen from these systems can then be used to develop a vaccine as above, and pigs, vaccinated with such products would develop protective immune responses against LA.

Vaccines against MSD can also be based on live preparations of LA. Since only young piglets and pregnant sows seem to be seriously affected by infection with LA, it is possible to use unattenuated LA, grown in pig lung macrophages, as vaccine for older piglets, or breeding gilts. In this way sows can be protected against MSD before they get pregnant, which results in protection against abortions and stillbirth, and against congenital infections of piglets. Also the maternal antibody that these vaccinated sows give to their offspring would protect their offspring against the disease.

Attenuated vaccines (modified-live-vaccines) against MSD can be made by serially passaging LA in pig lung macrophages, in lung macrophages of other species, or in other cell systems, or in other animals, such as rabbits, until it has lost its pathogenicity.

Live vaccines against MSD can also be made by recombinant DNA techniques through which the genome of LA, or parts thereof, is incorporated in vector systems such as vaccinia virus, herpesvirus, pseudorabies virus, adeno virus or other suitable vector systems that can so express LA antigen. Pigs, vaccinated with such live vector systems would then develop protective immune responses against LA.

Lelystad agent itself would be specifically suited to use as a live vector system. Foreign genes could be inserted in the genome of LA and could be expressing the corresponding protein during the infection of the macrophages. This cell, which is an antigen presenting cell, would process the foreign antigen and present it to B-lymfocytes and T-lymfocytes which will respond with the appropriate immune respons.

Since LA seems to be very cell specific and possibly also very species specific, this vector system might be a very safe system, which does not harm other cells or species.

SHORT DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the nucleotide sequence of the LV genome. The deduced amino acid sequence of the identified ORFs are shown. The methionines encoded by the (putative) ATG start sites are indicated in bold and putative N-glycosylation sites are underlined. Differences in the nucleotide and amino acid sequence, as identified by sequencing different cDNA clones, are shown. The nucleotide sequence of primer 25, which has been used in hybridization experiments (see Fig. 2 and section "results"), is underlined.

FIG. 2 shows the organization of the LV genome. The cDNA clones, which have been used for the determination of the nucleotide sequence, are indicated in the upper part of the

figure. The parts of the clones, which were sequenced, are indicated in black. In the lower part of the figure the ORFs, identified in the nucleotide sequence, and the subgenomic set of mRNAs, encoding these ORFs, are shown. The dashed lines in the ORFs represent alternative initiation sites (ATGs) of these ORFs. The leader sequence of the genomic and subgenomic RNAs is indicated by a solid box.

FIG. 3 shows the growth characteristics of LA:
- empty squares - titre of cell-free virus;
- solid squares - titre of cell-associated virus;
- solid line - percentage cytopathic effect (CPE).

MATERIALS AND METHODS

Sample collection

Samples and pigs were collected from farms where a herd epizootic of MSD seemed to occur. Important criteria for selecting the farm as being affected with MSD were: sows that were off feed, the occurrence of stillbirth and abortion, weak offspring, respiratory disease and death among young piglets. Samples from four groups of pigs have been investigated:
(1) tissue samples and an oral swab from affected piglets from the field (table 1A),
(2) blood samples and oral swabs from affected sows in the field (tables 1B and 4),
(3) tissue samples, nasal swabs and blood samples collected from specific-pathogen-free (SPF) pigs experimentally infected by contact with affected sows from the field or
(4) tissue samples, nasal swabs and blood samples collected from specific-pathogen-free (SPF) pigs experimentally infected by inoculation with blood samples of affected sows from the field (tables 2 and 5).

Sample preparation

Samples for virus isolation were obtained from piglets and sows which on clinical grounds were suspected to have MSD,

and from experimentally infected SPF pigs, sows and their piglets.

Tissue samples were cut on a cryostat microtome and sections were submitted for direct immunofluorescence testing (IFT) with conjugates directed against various pig pathogens.

10% Suspensions of tissues samples were prepared in Hank's BSS supplemented with antibiotics, and oral and nasal swabs were soaked in Hank's BSS supplemented with antibiotics. After one hour at room temperature, the suspensions were clarified for 10 min at 6000 g, and the supernatant was stored at -70°C for further use. Leucocyte fractions were isolated from EDTA or heparin blood as described earlier (Wensvoort and Terpstra, 1988), and stored at -70°C. Plasma and serum for virus isolation was stored at -70°C.

Serum for serology was obtained from sows suspected to be in the acute phase of MSD, a paired serum was taken 3-9 weeks later. Furthermore, sera were taken from the experimentally infected SPF pigs at regular intervals and colostrum and serum was taken from experimentally infected sows and their piglets. Sera for serology were stored at -20°C.

Cells

Pig lung macrophages were obtained from lungs of 5-6 weeks old SPF pigs or from lungs of adult SPF sows from the Central Veterinary Institute's own herd. The lungs were washed five to eight times with phosphate buffered saline (PBS). Each aliquot of washing fluid was collected and centrifuged for 10 min at 300 g. The resulting cell pellet was washed again in PBS and resuspended in cell culture medium (160 ml medium 199, supplemented with 20 ml 2.95% tryptose phosphate, 20 ml foetal bovine serum (FBS), and 4.5 ml 1.4% sodium bicarbonate) to a concentration of 4 x 10^7 cells/ml. The cell suspension was then slowly mixed with an equal volume of DMSO mix (6.7 ml of above medium, 1.3 ml FBS, 2 ml dimethylsulfoxide 97%), aliquoted in 2 ml ampoules and stored in liquid nitrogen.

Macrophages from one ampoule were prepared for cell culture by washing twice in Earle's MEM, and resuspended in 30 ml growth medium (Earle's MEM, supplemented with 10% FBS, 200 U/ml penicillin, 0.2 mg/ml streptomycine, 100 U/ml mycostatin, and 0.3 mg/ml glutamine). PK-15 cells (American Type Culture Collection, CCL33) and SK-6 cells (Kasza et al., 1972) were grown as described by Wensvoort et al. (1989). Secondary porcine kidney (PK2) cells were grown in Earle's MEM, supplemented with 10% FBS and the above antibiotics. All cells were grown in a cell culture cabinet at 37°C and 5% CO_2.

Virus isolation procedures.

Virus isolation was performed according to established techniques using PK2, PK-15 and SK-6 cells, and pig lung macrophages. The former three cells were grown in 25 ml flasks (Greiner), and inoculated with the test sample when monolayers had reached 70-80% confluency. Macrophages were seeded in 100 µl aliquots in 96-well microtiter plates (Greiner) or in larger volumes in appropriate flasks, and inoculated with the test sample within one hour after seeding. The cultures were observed daily for cytopathic effects (CPE), and frozen at -70°C when 50-70% CPE was reached or after five to ten days of culture. Further passages were made with freeze-thawed material of passage level 1 and 2 or higher. Some samples were also inoculated into nine to twelve day old embryonated hen eggs. Allantoic fluid was subinoculated two times using an incubation interval of three days and the harvest of the third passage was examined by haemagglutination at 4°C using chicken red blood cells, and by an ELISA specifically detecting nucleoprotein of influenza A viruses (De Boer et al., 1990).

Serology

Sera were tested in haemagglutinating inhibition tests (HAI) to study the development of antibody against haemagglutinating encephalitis virus (HEV), and swine influenza viruses H1N1 and H3N2 according to the protocol of

Masurel (1976). Starting dilutions of the sera in HAI were 1:9, after which the sera were diluted twofold.

Sera were tested in established enzyme-linked immunosorbent assays (ELISA) for antibodies against the glycoprotein gI of pseudorabies virus (PRV; Van Oirschot et al., 1988), porcine parvo virus (PPV; Westenbrink et al., 1989), bovine viral diarrhoea virus (BVDV; Westenbrink et al., 1986), and hog cholera virus (HCV; Wensvoort et al., 1988). Starting dilutions in the ELISA's were 1:5, after which the sera were diluted twofold.

Sera were tested for neutralizing antibodies against 30-300 $TCID_{50}$ of encephalomyocarditis viruses (EMCV), porcine enteroviruses (PEV), and Lelystad agent (LA) according to the protocol of Terpstra (1978). Starting dilutions of the sera in the serum neutralization tests (SNT) were 1:5, after which the sera were diluted twofold.

Sera were tested for binding with LA in an immunoperoxidase-monolayer assay (IPMA). Lelystad agent (LA; code: CDI-NL-2.91) was seeded in microtiter plates by adding 50 ml growth medium containing 100 $TCID_{50}$ LA to the wells of a microtiter plate containing freshly seeded lung macrophages. The cells were grown for two days and then fixed as described (Wensvoort, 1986). The test sera were diluted 1:10 in 0.15 M NaCl, 0.05% Tween 80, 4% horse serum, or diluted further in fourfold steps, added to the wells and then incubated for one hour at 37°C. Sheep-anti-pig immunoglobulins (Ig) conjugated to horse radish peroxidase (HRPO, DAKO) were diluted in the same buffer and used in a second incubation for one hour at 37°C, after which the plates were stained as described (Wensvoort et al., 1986). An intense red staining of the cytoplasm of infected macrophages indicated binding of the sera to LA.

Virus identification procedures

The identity of cytopathic isolates was studied by determining the buoyant density in CsCl, by estimating

particle size in negatively stained preparations through electron microscopy, by determining the sensitivity of the isolate to chloroform and by neutralizing the CPE of the isolate with sera with known specificity (Table 3). Whenever an isolate was specifically neutralized by a serum directed against a known virus, the isolate was considered to be a representative of this known virus.

Isolates that showed CPE on macrophage cultures were also studied by staining in IPMA with postinfection sera of pigs c 829 or b 822. The isolates were reinoculated on macrophage cultures and fixed at day 2 after inoculation before the isolate showed CPE. Whenever an isolate showed reactivity in IPMA with the postinfection sera of pigs c 829 or b 822, the isolate was considered to be a representative of the Lelystad agent. Representatives of the other isolates grown in macrophages or uninfected macrophages were also stained with these sera to check the specificity of the sera.

Further identification of Lelystad agent.

Lelystad agent was further studied by haemagglutination at 4°C and 37°C with chicken, guinea pig, pig, sheep, or human O red blood cells. SIV, subtype H3N2, was used as positive control in the haemagglutination studies.

The binding of pig antisera specifically directed against pseudorabies virus (PRV), transmissible gastroenteritis virus (TGE), porcine epidemic diarrhoea virus (PED), haemagglutinating encephalitis virus (HEV), African swine fever virus (ASFV), hog cholera virus (HCV) and swine influenza virus (SIV) type H1N1 and H3N2, of bovine antisera specifically directed against bovine herpes viruses type 1 and 4 (BHV 1 and 4), malignant catarrhal fever (MCF), parainfluenza virus 3 (PI3), bovine respiratory syncitial virus (BRSV) and bovine leukemia virus (BLV), and of avian antisera specifically directed against avian leukemia virus (ALV) and infectious bronchitis virus (IBV) was studied with

species-Ig specific HRPO conjugates in an IPMA on LA infected and uninfected pig lung macrophages as described above.

We also tested in IPMA antisera of various species directed against mumps virus, Sendai virus, canine distemper virus, rinderpest virus, measles virus, pneumonia virus of mice, bovine respiratory syncytial virus, rabies virus, foamy virus, maedi-visna virus, bovine and murine leukemia virus, human, feline and simian immunodeficiency virus, lymphocytic choriomeningitis virus, feline infectious peritonitis virus, mouse hepatitis virus, Breda virus, Hantaan virus, Nairobi sheep disease virus, Eastern, Western and Venezuelan equine encephalomyelitis virus, rubella virus, equine arteritis virus, lactic dehydrogenase virus, yellow fever virus, tick-born encephalitis virus and hepatitis C virus.

LA was blindly passaged in PK2, PK-15, and SK-6 cells, and in embryonated hen eggs. After two passages, the material was inoculated again into pig lung macrophage cultures for reisolation of LA.

LA was titrated in pig lung macrophages prior to and after passing through a 0.2 micron filter (Schleicher and Schuell). The LA was detected in IPMA and by its CPE. Titres were calculated according to Reed and Muench (1938).

We further prepared pig antisera directed against LA. Two SPF pigs (21 and 23) were infected intranasally with 10^5 $TCID_{50}$ of a fifth cell culture passage of LA. Two other SPF pigs (25 and 29) were infected intranasally with a fresh suspension of the lungs of an LA-infected SPF piglet containing 10^5 $TCID_{50}$ LA. Blood samples were taken at 0, 14, 28, and 42 days postinfection (dpi).

We further grew LA in porcine alveolar macrophages to determine its growth pattern over time. Porcine alveolar macrophages were seeded in F25 flasks (Greiner), infected with LA with a multiplicity of infection of 0.01 $TCID_{50}$ per cell. At 8, 16, 24, 32, 40, 48, 56, and 64 h after infection, one flask was examined and the percentage of CPE in relation to a noninfected control culture was determined. The culture medium

was then harvested and replaced with an equal volume of phosphate-buffered saline. The medium and the flask were stored at -70°C. After all cultures had been harvested, the LA titres were determined and expressed as log $TCID_{50}$ ml^{-1}.

The morphology of LA was studied by electronmicroscopy. LA was cultured as above. After 48 h, the cultures were freeze-thawed and centrifuged for 10 min at 6000 x g. An amount of 30 ml supernatant was then mixed with 0.3 ml LA-specific pig serum and incubated for 1.5 h at 37°C. After centrifugation for 30 min at 125,000 x g, the resulting pellet was suspended in 1% Seakem agarose ME in phosphate-buffered saline at 40°C. After coagulation, the agarose block was immersed in 0.8% glutaraldehyde and 0.8% osmiumtetroxide (Hirsch et al., 1968) in veronal/acetate buffer, pH 7.4 (230 mOsm/kg H_2O), and fixed by microwave irradiation. This procedure was repeated once with fresh fixative. The sample was washed with water, immersed in 1% uranyl acetate, and stained by microwave irradiation. Throughout all steps, the sample was kept at 0°C and the microwave (Samsung RE211D) was set at defrost for 5 min. Thin sections were prepared with standard techniques, stained with lead citrate (Venable et al., 1965), and examined in a Philips CM 10 electron microscope.

We further continued isolating LA from sera of pigs originating from cases of MSD. Serum samples originated from the Netherlands (field case the Netherlands 2), Germany (field cases Germany 1 and Germany 2; courtesy Drs. Berner, München and Nienhoff, Münster), and the United States [experimental case United States 1 (experiment performed with ATCC VR-2332; courtesy Drs. Collins, St. Paul and Chladek, St. Joseph), and field cases United States 2 and United States 2; courtesy Drs. van Alstine, West Lafayette and Slife, Galesburg]. All samples were sent to the "Centraal Diergeneeskundig Instituut, Lelystad" for LA diagnosis. All samples were used for virus isolation on porcine alveolar macrophages as described. Cytophatic isolates were passaged three times and identified

as LA by specific immunostaining with anti-LA post infection sera b 822 and c 829.

We also studied the antigenic relationships of isolates NL1 (the first LA isolate; code CDI-NL-2.91), NL2, GE1, GE2, US1, US2, and US3. The isolates were grown in macrophages as above and were tested in IPMA with a set of field sera and two sets of experimental sera. The sera were also tested in IPMA with uninfected macrophages.

The field sera were: Two sera positive for LV (TH-187 and TO-36) were selected from a set of LA-positive Dutch field sera. Twenty-two sera were selected from field sera sent from abroad to Lelystad for serological diagnosis. The sera originated from Germany (BE-352, BE-392 and NI-f2; courtesy Dr. Berner, München and Dr. Nienhoff, Münster), the United Kingdom (PA-141615, PA-141617 and PA-142440; courtesy Dr. Paton, Weybridge), Belgium (PE-1960; courtesy Prof. Pensaert, Gent), France (EA-2975 and EA-2985; courtesy Dr. Albina, Ploufragan), the United States (SL-441, SL-451, AL-RP9577, AL-P10814/33, AL-4994A, AL-7525, JC-MN41, JC-MN44 and JC-MN45; courtesy Dr. Slife, Galesburg, Dr. van Alstine, West Lafayette, and Dr. Collins, St. Paul), and Canada (RB-16, RB-19, RB-22 and RB-23; courtesy Dr. Robinson, Quebec).

The experimental sera were: The above described set of sera of pigs 21, 23, 25, and 29, taken at dpi 0, 14, 28, and 42. A set of experimental sera (obtained by courtesy of Drs. Chladek, St. Joseph, and Collins, St. Paul) that originated from four six-month-old gilts that were challenged intranasally with $10^{5.1}$ $TCID_{50}$ of the isolate ATCC VR-2332. Bloodsamples were taken from gilt 2B at 0, 20, 36, and 63 dpi; from gilt 9G at 0, 30, 44, and 68 dpi; from gilt 16W at 0, 25, 40, and 64 dpi; and from gilt 16Y at 0, 36, and 64 dpi.

To study by radio-immunoprecipitation assay (RIP; de Mazancourt et al., 1986) the proteins of LA in infected porcine alveolar macrophages, we grew LA-infected and uninfected macrophages for 16 hours in the presence of labeling medium containing ^{35}S-Cysteine. Then the labeled cells

were precipitated according to standard methods with 42 dpi post-infection sera of pig b 822 and pig 23 and with serum MN8 which was obtained 26 days after infecting a sow with the isolate ATCC VR-2332 (coutesy Dr. Collins, St. Paul). The precipitated proteins were analysed by electrophoresis in a 12% SDS-PAGE gel and visualized by fluorography.

To characterize the genome of LA, we extracted nuclear DNA and cytoplasmatic RNA from macrophage cultures that were infected with LA and grown for 24 h or were left uninfected. The cell culture medium was discarded, and the cells were washed twice with phosphate-buffered saline. DNA was extracted as described (Strauss, 1987). The cytoplasmic RNA was extracted as described (Favaloro et al., 1980), purified by centrifugation through a 5.7 M CsCl cushion (Setzer et al., 1980), treated with RNase-free DNase (Pharmacia), and analyzed in an 0.8% neutral agarose gel (Moormann and Hulst, 1988).

Cloning and Sequencing

To clone LV RNA, intracellular RNA of LV-infected porcine lung alveolar macrophages (10 µg) was incubated with 10 mM methylmercury hydroxide for 10 minutes at room temperature. The denatured RNA was incubated at 42°C with 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 70 mM KCl, 0.5 mM dATP, dCTP, dGTP and dTTP, 0.6 µg calf thymus oligonucleotide primers pd(N)6 (Pharmacia) and 300 units of Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) in a total volume of 100 µl. 20 mM EDTA was added after 1 hr; the reaction mixture was then extracted with phenol/chloroform, passed through a Sephadex G50 column and precipitated with ethanol.

For synthesis of the second cDNA strand, DNA polymerase I (Boehringer) and RNase H (Pharmacia) were used (Gübler and Hoffman, 1983). To generate blunt ends at the termini, double-stranded cDNA was incubated with T4 DNA polymerase (Pharmacia) in a reaction mixture which contained 0.05 mM deoxynucleotide-triphosphates. Subsequently, cDNA was fractionated in a 0.8%

neutral agarose gel (Moormann and Hulst, 1988). Fragments of 1 to 4 kb were electroeluted, ligated into the SmaI site of pGEM-4Z (Promega), and used for transformation of Escherichia coli strain DH5α (Hanahan, 1985). Colony filters were hybridized with a ^{32}P-labelled single-stranded cDNA probe. The probe was reverse transcribed from LV RNA which had been fractionated in a neutral agarose gel (Moormann and Hulst, 1988). Before use the single stranded DNA probe was incubated with cytoplasmic RNA from mock-infected lung alveolar macrophages.

The relationship between LV cDNA clones was determined by restriction enzyme analysis and by hybridization of Southern blots of the digested DNA with nick-translated cDNA probes (Sambrook et al., 1989).

To obtain the 3' end of the viral genome, we constructed a second cDNA library, using oligo $(dT)_{12-18}$ and a 3' LV specific oligonucleotide that was complementary to the minus-strand viral genome as a primer in the first-strand reaction. The reaction conditions for first- and second-strand synthesis were identical to those described above. This library was screened with virus-specific 3' end oligonucleotide probes.

Most part (> 95%) of the cDNA sequence was determined with an Automated Laser Fluorescent A.L.F.™ DNA sequencer from Pharmacia LKB. Fluorescent oligonucleotide primer directed sequencing was performed on double-stranded DNA using the AutoRead™ Sequencing Kit (Pharmacia) essentially according to procedures C and D described in the Autoread™ Sequencing Kit protocol. Fluorescent primers were prepared with FluorePrime™ (Pharmacia). The remaining part of the sequence was determined via double-stranded DNA sequencing using oligonucleotide primers in conjunction with a T7 polymerase based sequencing kit (Pharmacia) and α-^{32}S-dATP (Amersham). Sequence data were analysed using the sequence analysis programs PCGENE (Intelligenetics, Inc, Mountain View, USA) and FASTA (Pearson and Lipman, 1988).

Experimental reproduction of MSD.

Fourteen conventionally reared pregnant sows that were pregnant for 10-11 weeks were tested for antibody against LA in the IPMA. All were negative. Then two groups of four sows were formed and brought to the CVI. At week 12 of gestation, these sows were inoculated intranasally with 2 ml LA (passage level 3, titre $10^{4.8}$ $TCID_{50}$/ml). Serum and EDTA blood samples were taken at day 10 after inoculation. Food intake, rectal temperature, and other clinical symptoms were observed daily. At farrowing, the date of birth and the number of dead and living piglets per sow were recorded, and samples were taken for virus isolation and serology.

RESULTS

Immunofluorescence

Tissue sections of pigs with MSD were stained in an IFT with FITC-conjugates directed against African swine fever virus, hog cholera virus, pseudorabies virus, porcine parvo virus, porcine influenza virus, encephalomyocarditis virus and Chlamydia psittaci. The sections were stained, examined by fluorescent microscopy and all were found negative.

Virus isolation from piglets from MSD affected farms.

Cytopathic isolates were detected in macrophage cultures inoculated with tissue samples of MSD affected, two-to-ten day old piglets. Sixteen out of 19 piglets originating from five different farms were positive (Table 1A). These isolates all reacted in IPMA with the post-infection serum of pig c 829, whereas non-inoculated control cultures did not react. The isolates therefore were representatives of LA. One time a cytopathic isolate was detected in an SK-6 cell culture inoculated with a suspension of an oral swab from a piglet from a sixth farm (farm VE) (Table 1A). This isolate showed characteristics of the picorna viridae and was neutralized by serum specific for PEV 2, therefore the isolate was identified

as PEV 2 (Table 3). PK2, PK-15 cells and hen eggs inoculated with samples from this group remained negative throughout.

Virus isolation from sows from MSD affected farms.

Cytopathic isolates were detected in macrophage cultures inoculated with samples of MSD affected sows. 41 out of 63 sows originating from 11 farms were positive (Table 1B). These isolates all reacted in IPMA with the post-infection serum of pig b 822 and were therefore representatives of LA. On one occasion a cytopathic isolate was detected in a PK2 cell culture inoculated with a suspension of a leucocyte fraction of a sow from farm HU (Table 1B). This isolate showed characteristics of the picorna viridae and was neutralized by serum specific for EMCV, therefore the isolate was identified as EMCV (Table 3). SK-6, PK-15 cells and hen eggs inoculated with samples from this group remained negative.

Virus isolation from SPF pigs kept in contact with MSD affected sows.

Cytopathic isolates were detected in macrophage cultures inoculated with samples of SPF pigs kept in contact with MSD affected sows. Four of the 12 pigs were positive (Table 2). These isolates all reacted in IPMA with the post-infection serum of pig c 829 and of pig b 822 and were therefore representatives of LA. Cytopathic isolates were also detected in PK2, PK-15 and SK-6 cell cultures inoculated with samples of these SPF pigs. Seven of the 12 pigs were positive (Table 2), these isolates were all neutralized by serum directed against PEV 7. One of these seven isolates was studied further and other characteristics also identified the isolate as PEV 7 (Table 3).

Virus isolation from SPF pigs inoculated with blood of MSD affected sows.

Cytopathic isolates were detected in macrophage cultures inoculated with samples of SPF pigs inoculated with blood of

MSD affected sows. Two out of the eight pigs were positive (Table 2). These isolates all reacted in IPMA with the post-infection serum of pig c 829 and of pig b 822 and were therefore representatives of LA. PK2, SK-6 and PK-15 cells inoculated with samples from this group remained negative.

Summarizing, four groups of pigs were tested for the presence of agents that could be associated with mystery swine disease (MSD).

In group one, MSD affected piglets, the Lelystad agent (LA) was isolated from 16 out of 20 piglets; one time PEV 2 was isolated.

In group two, MSD affected sows, the Lelystad agent was isolated from 41 out of 63 sows; one time EMCV was isolated. Furthermore, 123 out of 165 MSD affected sows seroconverted to the Lelystad agent, as tested in the IPMA. Such massive seroconversion was not demonstrated against any of the other viral pathogens tested.

In group three, SPF pigs kept in contact with MSD affected sows, LA was isolated from four of the 12 pigs; PEV 7 was isolated from seven pigs. All 12 pigs pigs seroconverted to LA and PEV 7.

In group four, SPF pigs inoculated with blood of MSD affected sows, the LA was isolated from two pigs. All eight pigs seroconverted to LA.

Serology of sows from MSD affected farms.

Paired sera from sows affected with MSD were tested against a variety of viral pathogens and against the isolates obtained during this study (Table 4). An overwhelming antibody respons directed against LA was measured in the IPMA (75% of the sows seroconverted, in 23 out of the 26 farms seroconversion was found), whereas with none of the other viral pathogens a clear pattern of seroconversion was found. Neutralizing antibody directed against LA was not detected.

Serology of SPF pigs kept in contact with MSD affected sows.

All eight SPF pigs showed an antibody respons in the IPMA against LA (Table 5). None of these sera were positive in the IPMA performed on uninfected macrophages. None of these sera were positive in the SNT for LA. The sera taken two weeks after contact had all high neutralizing antibody titres (>1280) against PEV 7, whereas the pre-infection sera were negative (<10), indicating that all pigs had also been infected with PEV 7.

Serology of SPF pigs inoculated with blood of MSD affected sows.

All eight SPF pigs showed an antibody response in the IPMA against LA (Table 5). None of these sera were positive in the IPMA performed on uninfected macrophages. None of these sera were positive in the SNT for LA. The pre- and two weeks post-inoculation sera were negative (<10) against PEV 7.

Further identification of Lelystad agent.

LA did not haemagglutinate with chicken, guinea pig, pig, sheep, or human O red blood cells.

LA did not react in IPMA with sera directed againts PRV, TGE, PED, ASFV, etc.

After two blind passages, LA did not grow in PK2, PK-15, or SK-6 cells, or in embryonated hen eggs, inoculated through the allantoic route.

LA was still infectious after it was filtred through a 0.2 micron filter, titres before and after filtration were $10^{5.05}$ and $10^{5.3}$ $TCID_{50}$ as detected by IPMA.

Growth curve of LA (see figure 3). Maximum titres of cell-free virus were approximately $10^{5.5}$ $TCID_{50}$ ml^{-1} from 32-48 h after inoculation. After that time the macrophages were killed by the cytopathic effect of LA.

Electronmicroscopy. Clusters of spherical LA particles were found. The particles measured 45-55 nm in diameter and contained a 30-35 nm nucleocapsid that was surrounded by a

lipid bilayer membrane. LA particles were not found in infected cultures that were treated with negative serum or in negative control preparations.

Isolates from the Netherlands, Germany, and the United States. All seven isolates were isolated in porcine alveolar macrophages and passaged three to five times. All isolates caused a cytopathic effect in macrophages and could be specifically immunostained with anti-LA sera b 822 and the 42 dpi serum 23. The isolates were named NL2, GE1, GE2, US1, US2, and US3.

Antigenic relationships of isolates NL1, NL2, GE1, GE2, US1, US2, and US3. None of the field sera reacted in IPMA with uninfected macrophages but all sera contained antibodies directed against one or more of the seven isolates (Table 7). None of the experimental sera reacted in IPMA with uninfected macrophages, and none of the 0 dpi experimental sera reacted with any of the seven isolates in IPMA (Table 8). All seven LA isolates reacted with all or most of the sera from the set of experimental sera of pigs 21, 23, 25, and 29, taken after 0 dpi. Only the isolates US1, US2, and US3 reacted with all or most of the sera from the set of experimental sera of gilts 2B, 9G, 16W, and 16Y, taken after 0 dpi.

Radioimmunoprecipitation studies. Seven LA-specific proteins were detected in LA-infected macrophages but not in uninfected macrophages precipitated with the 42 dpi sera of pigs b 822 and 23. The proteins had estimated molecular weights of 65, 39, 35, 26, 19, 16, and 15 kilodalton. Only two of these LA-specific proteins, of 16 and 15 kilodalton, were also precipitated by the 26 dpi serum MN8.

Sequence and organization of the genome of LV

The nature of the genome of LV was determined by analyzing DNA and RNA from infected porcine lung alveolar macrophages. No LV-specific DNA was detected. However, we did detect LV-specific RNA. In a 0.8% neutral agarose gel LV RNA migrated slightly slower than a preparation of hog cholera

virus RNA of 12.3 kb (Moormann et al., 1990) did. Although no accurate size determination can be performed in neutral agarose gels, it was estimated that the LV-specific RNA is about 14.5 to 15.5 kb in length.

To determine the complexity of the LV-specific RNAs in infected cells and to establish the nucleotide sequence of the genome of LV, we prepared cDNA from RNA of LV-infected porcine lung alveolar macrophages and selected and mapped LV-specific cDNA clones as described under Materials and Methods. The specificity of the cDNA clones was reconfirmed by hybridizing specific clones, located throughout the overlapping cDNA sequence, to Northern blots carrying RNA of LV-infected and uninfected macrophages. Remarkably, some of the cDNA clones hybridized with the 14.5 to 15.5 kb RNA detected in infected macrophages only, whereas others hybridized with the 14.5 to 15.5 kb RNA as well as with a panel of 4 or 5 RNAs of lower molecular weight (estimated size, 1 to 4 kb). The latter clones were all clustered at one end of the cDNA map and covered about 4 kb of DNA. These data suggested that the genome organization of LV may be similar to that of coronaviridae (Spaan et al., 1988), Berne virus (BEV; Snijder et al., 1990b), a torovirus, and EAV (de Vries et al., 1990), i.e. besides a genomic RNA there are subgenomic mRNAs which form a nested set which is located at the 3' end of the genome. This assumption was confirmed when sequences of the cDNA clones became available and specific primers could be selected to probe the blots with. A compilation of the hybridization data obtained with cDNA clones and specific primers, which were hybridized to Northern blots carrying the RNA of LV-infected and uninfected macrophages, is shown in figure 2. Clones 12 and 20 which are located in the 5' part and the centre of the sequence respectively hybridize to the 14.5 to 15.5 kb genomic RNA detected in LV-infected cells only. Clones 41 and 39, however, recognize the 14.5 to 15.5 kb genomic RNA and a set of 4 and 5 RNAs of lower molecular weight, respectively. The most instructive and conclusive

hybridization pattern, however, was obtained with primer 25, which is located at the ultimate 5' end in the LV sequence (compare Fig. 1). Primer 25 hybridized to a panel of 7 RNAs, with an estimated molecular weight ranging in size from 0.7 to 3.3 kb (subgenomic mRNAs), as well as the genomic RNA. The most likely explanation for the hybridization pattern of primer 25 is that 5' end genomic sequences, the length of which is yet unknown, fuse with the body of the mRNAs which are transcribed from the 3' end of the genome. In fact, the hybridization pattern obtained with primer 25 suggests that 5' end genomic sequences function as a so called "leader sequence" in subgenomic mRNAs. Such a transcription pattern is a hallmark of replication of coronaviridae (Spaan et al., 1988), and of EAV (de Vries et al., 1990).

The only remarkable discrepancy between LV and EAV which could be extracted from the above data is that the genome size of LV is about 2.5 kb larger than that of EAV.

The consensus nucleotide sequence of overlapping cDNA clones is shown in figure 1. The length of the sequence is 15,088 basepairs, which is in good agreement with the estimated size of the genomic LV RNA.

Since the LV cDNA library was made by random priming of the reverse transcriptase reaction with calf thymus pd(N)6 primers, no cDNA clones were obtained which started with a poly-A stretch at their 3' end. To clone the 3' end of the viral genome, we constructed a second cDNA library, using oligo (dT) and primer 39U183R in the reverse transcriptase reaction. Primer 39U183R is complementary to LV minus-strand RNA, which is likely present in a preparation of RNA isolated from LV-infected cells. This library was screened with virus-specific probes (nick-translated cDNA clone 119 and oligonucleotide 119R64R), resulting in the isolation of five additional cDNA clones (e.g., cDNA clone 151, Fig. 2). Sequencing of these cDNA clones revealed that LV contains a 3' poly(A) tail. The length of the poly(A) tail varied between the various cDNA clones, but its maximum length was twenty

nucleotides. Besides clone 25 and 155 (Fig. 2), four additional cDNA clones were isolated at the 5' end of the genome, which were only two to three nucleotides shorter than the ultimate 5' nucleotide shown in figure 1. Given this finding and given the way cDNA was synthesized, we assume to be very close to the 5' end of the sequence of LV genomic RNA.

Nearly 75% of the genomic sequence of LV encodes ORF 1A and ORF 1B. ORF 1A probably initiates at the first AUG (nucleotide position 212, Fig. 1) encountered in the LV sequence. The C-terminus of ORF 1A overlaps the putative N-terminus of ORF 1B over a small distance of 16 nucleotides. It thus seems that translation of ORF 1B proceeds via ribosomal frameshifting, a hallmark of the mode of translation of the polymerase or replicase gene of coronaviruses (Boursnell et al., 1987; Bredenbeek et al. 1990) and the torovirus BEV (Snijder et al., 1990a). The characteristic RNA pseudoknot structure which is predicted to be formed at the site of the ribosomal frameshifting is also found at this location in the sequence of LV (results not shown).

ORF 1B encodes an amino acid sequence of nearly 1400 residues which is much smaller than ORF 1B of the coronaviruses MHV and IBV (about 3,700 amino acid residues; Bredenbeek et al., 1990; Boursnell et al., 1987) and BEV (about 2,300 amino acid residues; Snijder et al., 1990a). Characteristic features of the ORF 1B product of members of the superfamily of coronaviridae like the replicase motif and the Zinc finger domain can also be found in ORF 1B of LV (results not shown).

Whereas ORF 1A and ORF 1B encode the viral polymerase and therefore are considered to encode a non-structural viral protein, ORFs 2 to 7 are believed to encode structural viral proteins.

The products of ORFs 2 to 6 all show features reminiscent of membrane (envelope) associated proteins. ORF 2 encodes a protein of 249 amino acids containing two predicted N-linked glycosylation sites (Table 9). At the N-terminus a hydrophobic

sequence, which may function as a so called signal sequence, is identified. The C-terminus also ends with a hydrophobic sequence which in this case may function as a transmembrane region which anchors the ORF 2 product in the viral envelope membrane.

ORF 3 may initiate at the AUG starting at nucleotide position 12394 or at the AUG starting at nucleotide position 12556 and then encodes proteins of 265 and 211 amino acids respectively. The protein of 265 residues contains seven putative N-linked glycosylation sites, whereas the protein of 211 residues contains four (Table 9). At the N-terminus of the protein of 265 residues a hydrophobic sequence is identified.

Judged by hydrophobicity analysis, the topology of the protein encoded by ORF 4 is similar to that encoded by ORF 2 if the product of ORF 4 initiates at the AUG starting at nucleotide position 12936. However, ORF 4 may also initiate at two other AUG codons (compare figures 1 and 2) starting at positions 12981 and 13068 in the sequence respectively. Up to now it is unclear which startcodon is used. Depending on the startcodon used, ORF 4 may encode proteins of 183 amino acids containing four putative N-linked glycosylation sites, of 168 amino acids containing four putative N-linked glycosylation sites, or of 139 amino acids containing three putative N-linked glycosylation sites (Table 9).

ORF 5 is predicted to encode a protein of 201 amino acids having two putative N-linked glycosylation sites (Table 9). A characteristic feature of the ORF 5 product is the internal hydrophobic sequence between amino acid 108 to amino acid 132.

Analysis for membrane spanning segments and hydrophilicity of the product of ORF 6 shows that it contains three transmembrane spanning segments in the N-terminal 90 amino acids of its sequence. This remarkable feature is also a characteristic of the small envelope glycoprotein M or E1 of several coronaviruses e.g. Infectious Bronchitis Virus (IBV; Boursnell et al., 1984) and Mouse Hepatitis Virus (MHV: Rottier et al., 1986). It is therefore predicted that the

protein encoded by ORF 6 has a membrane topology analogous to that of the M or E1 protein of coronaviruses (Rottier et al., 1986). A second characteristic of the M or E1 protein is a so called surface helix which is located immediately adjacent to the presumed third transmembrane region. This sequence of about 25 amino acids which is very well conserved among coronaviruses is also recognized, although much more degenerate, in LV. Yet we predict the product of LV ORF 6 to have an analogous membrane associated function as the coronavirus M or E1 protein. Furthermore, the protein encoded by ORF 6 showed a strong similarity (53% identical amino acids) with VpX (Godeny et al., 1990) of LDV.

The protein encoded by ORF 7 has a length of 128 amino acid residues (Table 9) which is 13 amino acids longer than Vp1 of LDV (Godeny et al., 1990). Yet a significant similarity (43% identical amino acids) was observed between the protein encoded by ORF 7 and Vp1. Another shared characteristic between the product of ORF 7 and Vp1 is the high concentration of basic residues (Arg, Lys and His) in the N-terminal half of the protein. Up to amino acid 55 the LV sequence contains 26% Arg, Lys and His. This finding is fully in line with the proposed function of the ORF 7 product or Vp1 (Godeny et al., 1990), namely encapsidation of the viral genomic RNA. On the basis of above data, we propose the LV ORF 7 product to be the nucleocapsid protein N of the virus.

A schematic representation of the organization of the LV genome is shown in figure 2. The map of overlapping clones used to determine the sequence of LV is shown in the top panel. A linear compilation of this map indicating the 5' and 3' end of the nucleotide sequence of LV, shown in figure 1, including a division in kilobases is shown below the map of cDNA clones and allows the positioning of these clones in the sequence. The position of the ORFs identified in the LV genome is indicated below the linear map of the LV sequence. The bottom panel shows the nested set of subgenomic mRNAs and the position of these RNAs relative to the LV sequence.

In line with the translation strategy of coronavirus, torovirus and arterivirus subgenomic mRNAs it is predicted that ORFs 1 to 6 are translated from the unique 5' end of their genomic or mRNAs. This unique part of the mRNAs is considered to be that part of the RNA that is obtained when a lower molecular weight RNA is "subtracted" from the higher molecular weight RNA which is next in line. Although RNA 7 forms the 3' end of all the other genomic and subgenomic RNAs, and thus does not have a unique region, it is believed that ORF 7 is only translated from this smallest sized mRNA. The "leader sequence" at the 5' end of the subgenomic RNAs is indicated with a solid box. The length of this sequence is about 200 bases, but the precise site of fusion with the body of the genomic RNAs still has to be determined.

Experimental reproduction of MSD

Eight pregnant sows were inoculated with LA and clinical signs of MSD such as inappetance and reproductive losses were reproduced in these sows. From day four to day 10-12 post-inoculation (p.i.), all sows showed a reluctance to eat. None of the sows had elevated body temperatures. Two sows had bluish ears at day 9 and 10 p.i. In Table 6 the day of birth and the number of living and dead piglets per sow is given. LA was isolated from 13 of the born piglets.

Table 1.
Description and results of virus isolation of field samples.

A Samples of piglets suspected of infection with MSD.

farm	number of pigs	age days	material used	results*
RB	5	2	lung, tonsil, and brains	5 x LA
DV	4	3	lung, brains, pools of kidney, spleen	3 x LA
TH	3	3-5	lung, pools of kidney, tonsil	3 x LA
DO	3	10	lung, tonsil	2 x LA
ZA	4	1	lung, tonsil	3 x LA
VE	1	?	oral swab	1 x PEV 2
TOTAL	20			16 x LA, 1 x PEV 2

B Samples of sows suspected of infection with MSD.

farm	number of sows	material used	results
TH	2	plasma and leucocytes	1 x LA
HU	5	plasma and leucocytes	2 x LA, 1 X EMCV
TS	10	plasma and leucocytes	6 x LA
HK	5	plasma and leucocytes	2 x LA
LA	6	plasma and leucocytes	2 x LA
VL	6	serum and leucocytes	5 x LA
TA	15	serum	11 x LA
LO	4	plasma and leucocytes	2 x LA
JA	8	plasma and leucocytes	8 x LA
VD	1	plasma and leucocytes	1 x LA
VW	1	serum	1 x LA
TOTAL	63		41 x LA, 1 x EMCV

* Results are given as the number of pigs from which the isolation was made. Sometimes the isolate was detected in more then one sample per pig.
LA = Lelystad agent
PEV 2 = porcine entero virus type 2
EMCV = encephalomyocarditis virus

Table 2.
Description and results of virus isolation of samples of pigs with experimentally induced infections.

sow	pig@	material used	results*
A (LO) #	c 835	lung, tonsil	2 x LA
	c 836	nasal swabs	2 x PEV 7
	c 837	nasal swabs	
B (JA)	c 825	lung, tonsil	
	c 821	nasal swabs	1 x PEV 7
	c 823	nasal swabs	4 x PEV 7
C (JA)	c 833	lung, tonsil	1 x LA, 1 x PEV 7
	c 832	nasal swabs	2 x PEV 7
	c 829	nasal swabs, plasma and leucocytes	3 x LA, 2 x PEV 7
D (VD)	c 816	lung, tonsil	
	c 813	nasal swabs	1 x LA
	c 815	nasal swabs	1 x PEV 7
TOTAL isolates from contact pigs			7 x LA, 13 x PEV 7
A	b 809	nasal swabs	
	b 817	nasal swabs	
B	b 818	nasal swabs, plasma and leucocytes	1 x LA
	b 820	nasal swabs	
C	b 822	nasal swabs	
	b 826	nasal swabs	
D	b 830	nasal swabs	1 x LA
	b 834	nasal swabs	
TOTAL isolates from blood inoculated pigs			2 x LA

@ SPF pigs were either kept in contact (c) with a sow suspected to be infected with MSD, or were given 10 ml EDTA blood (b) of that sow intramuscularly at day 0 of the experiment. Groups of one sow and three SPF pigs (c) were kept in one pen, and all four of these groups were housed in one stable. At day 6, one SPF pig in each group was killed and tonsil and lungs were used for virus isolation. The four groups of SPF pigs inoculated with blood (b) were housed in four other pens in a separate stable. Nasal swabs of the SPF pigs were taken at day 2, 5, 7 and 9 of the experiment, and EDTA blood for virus isolation from plasma and leucocytes was taken whenever a pig had fever.

* Results are given as number of isolates per pig.
LA = Lelystad agent
PEV 7 = porcine entero virus type 7
In brackets the initials of the farm of origin of the sow are given.

Table 3.
Identification of viral isolates

origin and cell culture	buoyant[1] density in CsCl	particle[2] size in EM (nm)	sens[3]. to chloroform	neutralized by[4] serum directed against (titre)
leucocytes sow farm HU PK-15, PK2, SK6	1.33 g/ml	28-30	not sens.	EMCV (1280)
oral swab piglet farm VE SK6	ND	28-30	not sens.	PEV 2 (> 1280)
nasal swabs, tonsil SPF pigs CVI PK-15, PK2, SK6	ND	28-30	not sens.	PEV 7 (> 1280)
various samples various farms pig lung macrophages	1.19 g/ml	pleomorf	sens.	none (all < 5)

1) Buoyant density in preformed lineair gradients of CsCl in PBS was determined according to standard techniques (Brakke; 1967). Given is the density where the peak of infectivity was found.
2) Infected and noninfected cell cultures of the isolate under study were freeze-thawed. Cell lysates were centrifuged for 30 min at 130,000 g, the resulting pellet was negatively stained according to standard techniques (Brenner and Horne; 1959), and studied with a Philips CM 10 electron microscope. Given is the size of particles that were present in infected and not present in non-infected cultures.
3) Sensitivity to chloroform was determined according to standard techniques (Grist, Ross, and Bell; 1974).
4) Hundred to 300 $TCID_{50}$ of isolates were mixed with varying dilutions of specific antisera and grown in the appropriate cell system until full CPE was observed. Sera with titres higher then 5 were retested, and sera which blocked with high titres the CPE were considered specific for the isolate.
The isolates not sensitive to chloroform were tested with sera specifically directed against porcine entero viruses (PEV) 1 to 11 (courtesy Dr. Knowles, Pirbright, UK), against encephalomyocarditis virus (EMCV; courtesy Dr. Ahl, Tübingen, Germany), against porcine parvo virus, and against swine vesicular disease.
The isolate (code: CDI-NL-2.91) sensitive to chloroform was tested with antisera specifically directed against pseudo-rabies virus, bovine herpes virus 1, bovine herpes virus 4, malignant catarrhal virus, bovine viral diarrhoea virus, hog cholera virus, swine influenza virus H1N1 and H3N2, parainfluenza 3 virus, bovine respiratory syncitial virus, transmissible gastroenteritis virus, porcine epidemic diarrhoea virus, haemaglutinating encephalitis virus, infectious bronchitis virus, bovine leukemia virus, avian leukemia virus, maedi-visna virus, and with the experimental sera obtained from the SPF-pigs (see Table 5).

Table 4.
Results of serology of paired field sera taken from sows suspected to have MSD. Sera were taken in the acute phase of the disease and 3-9 weeks later. Given is the number of sows which showed a fourfold or higher rise in titre/number of sows tested.

Farm	Interval[i] in weeks	HAI			ELISA			
		HEV	H1N1	H3N2	PRV	PPV	BVDV	HCV
TH	3	0/6	0/6	0/6	0/6	0/6	0/5	0/6
RB	5	0/13	1/13	0/13	1/9	0/7	0/6	0/9
HU	4	0/5	0/5	3/5	0/5	0/5	0/5	0/5
TS	3	1/10	0/10	0/10	0/10	0/10	0/4	0/10
VL	3	0/5	0/5	0/5	0/5	1/5	0/5	0/5
JA	3	0/11	1/11	3/11	0/11	2/11	0/11	0/11
WE	4	1/6	1/6	1/6	3/7	3/7	0/7	0/7
GI	4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
SE	5	0/8	0/8	0/8	0/8	0/6	0/3	0/8
KA	5	0/1	0/1	0/1	0/1	0/1	ND	0/1
HO	3	1/6	0/5	1/6	0/6	0/6	0/6	0/6
NY	4	0/5	1/5	1/5	0/3	0/4	0/2	0/4
JN	3	0/10	5/10	0/10	0/10	1/10	0/10	0/10
KO[f]	3	1/10	0/10	0/10	0/10	2/10	0/10	0/10
OE	9	ND	ND	ND	0/6	0/6	0/6	0/6
LO	6	ND	ND	ND	0/3	0/3	0/2	0/3
WI	4	ND	ND	ND	0/1	1/1	0/1	0/3
RR	3	ND	ND	ND	1/8	0/8	0/8	0/8
RY	4	ND	ND	ND	0/3	0/4	0/3	0/4
BE	5	ND	ND	ND	0/10	0/10	0/10	0/10
BU	3	ND	ND	ND	1/6	0/6	0/6	0/6
KR	3	ND	ND	ND	1/4	0/4	0/4	0/4
KW	5	ND	ND	ND	0/10	0/10	0/10	0/10
VR	5	ND	ND	ND	1/6	0/6	0/6	0/6
HU	4	ND	ND	ND	1/4	0/3	0/3	0/4
ME	3	ND	ND	ND	0/5	1/5	0/5	0/5
total negative[n]		19	41	29	97	16	140	165
total positive[p]		77	48	62	55	131	1	0
total sero-converted[s]		4	10	9	9	11	0	0
total tested		100	99	100	161	158	141	165

The sera were tested in haemagglutinating inhibition (HAI) tests for the detection of antibody against haemagglutinating encephalitis virus (HEV), and swine influenza viruses H1N1 and H3N2, in enzyme-linked-immuno sorbent assays (ELISA) for the detection of antibody against the glycoprotein gI of pseudorabies virus (PRV), against porcine parvo virus (PPV), bovine viral diarrhoea virus (BVDV), and hog cholera virus (HCV).

Table 4 - continued

Farm	Interval in weeks	SNT EMCV	EMCVi	PEV2	PEV2i	PEV7	PEV7i	LA	IPMA LA
TH	3	0/6	0/6	0/5	0/5	0/6	0/5	0/6	6/6
RB	5	1/7	1/9	0/6	2/6	1/8	0/6	0/13	7/9
HU	4	ND	0/5	0/5	0/5	ND	0/5	0/5	5/5
TS	3	0/10	0/10	0/7	0/4	0/10	0/7	ND	10/10
VL	3	ND	ND	1/5	0/5	ND	0/5	ND	5/5
JA	3	0/11	0/11	0/11	0/11	1/11	2/11	0/5	8/11
WE	4	1/7	1/6	1/6	1/7	1/7	1/7	0/7	7/7
GI	4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4
SE	5	0/8	0/8	0/6	1/8	0/8	1/5	0/8	6/8
KA	5	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
HO	3	0/6	0/6	0/6	0/6	0/6	0/6	0/6	4/6
NY	4	0/4	0/4	0/2	0/2	0/4	0/3	0/4	4/4
JN	3	0/10	0/10	1/10	0/9	0/10	0/10	0/10	5/10
KO[f]	3	0/10	0/10	2/10	2/10	1/10	3/10	ND	8/10
OE	9	0/6	0/6	1/6	1/5	ND	1/6	ND	4/6
LO	6	0/3	0/3	0/3	0/3	0/3	0/3	ND	3/3
WI	4	ND	ND	0/1	0/1	ND	0/1	ND	0/3
RR	3	0/8	1/8	0/8	0/8	0/8	0/8	ND	8/8
RY	4	0/4	ND	0/4	0/1	ND	1/4	ND	1/4
BE	5	ND	ND	0/10	0/10	ND	1/10	ND	0/10
BU	3	ND	ND	0/6	0/6	ND	0/6	ND	6/6
KR	3	ND	ND	0/4	0/4	ND	0/4	ND	1/4
KW	5	ND	ND	0/10	0/10	ND	1/10	ND	10/10
VR	5	ND	ND	0/6	1/6	ND	0/6	ND	6/6
HU	4	ND	ND	0/3	0/4	ND	0/3	ND	3/4
ME	3	ND	ND	0/5	0/5	ND	0/5	ND	2/5
total neg.[n]		15	29	0	0	2	1	69	15
total pos.[p]		88	74	144	138	90	136	0	27
total sero-converted[s]		2	3	6	8	4	10	0	123
total tested		105	107	150	146	96	147	69	165

The sera were tested in serum neutralization tests (SNT) for the detection of neutralizing antibody directed against encephalomyocarditis virus (EMCV), the isolated (i) EMCV, porcine entero viruses (PEV) 2 and 7 and the PEV isolates (i), and against the Lelystad agent (LA), and were tested in an immuno-peroxidase-monolayer-assay (IPMA) for the detection of antibody directed against the Lelystad agent (LA).

[f] fattening pigs. [i] time between sampling of the first and second serum. [n] total number of pigs of which the first serum was negative in the test under study, and of which the second serum was also negative or showed a less then fourfold rise in titre. [p] total number of pigs of which the first serum was positive and of which the second serum showed a less then fourfold rise in titre. [s] total number of pigs of which the second serum had a fourfold or higher titre then the first serum in the test under study. ND = not done.

Table 5.
Development of antibody directed against Lelystad agent as measured by IPMA.

A contact pigs	serum titres in IPMA				
Weeks post contact:	0	2	3	4	5
Pig					
c 836	0	10	640	640	640
c 837	0	10	640	640	640
c 821	0	640	640	640	640
c 823	0	160	2560	640	640
c 829	0	160	640	10240	10240
c 832	0	160	640	640	2560
c 813	0	640	2560	2560	2560
c 815	0	160	640	640	640

B blood inoculated pigs	serum titres in IPMA				
Weeks post inoculation:	0	2	3	4	6
Pig					
b 809	0	640	2560	2560	2560
b 817	0	160	640	640	640
b 818	0	160	640	640	640
b 820	0	160	640	640	640
b 822	0	640	2560	2560	10240
b 826	0	640	640	640	10240
B 830	0	640	640	640	2560
B 834	0	160	640	2560	640

See Table 2 for description of the experiment. All pigs were bled at regular intervals and all sera were tested in an immuno-peroxidase-monolayer-assay (IPMA) for the detection of antibody directed against the Lelystad agent (LA).

Table 6.
Experimental reproduction of MSD.

sow	length of gestation	No. of piglets at birth alive (number Ab pos)[2]	dead	No. of deaths week 1	LA[1] in piglets born dead	died in week 1
52	113	12(5)	3(2)	6	2	4
965	116	3(0)	9(3)	2	4	
997	114	9(0)	1(0)	0		
1305	116	7(0)	2(0)	1		
134	109	4(4)	7(4)	4	3	
941	117	7	10			
1056	113	7(1)	3(0)	4		
1065	115	9	2			

1) LA was isolated from lung, liver, spleen, kidney, or ascitic fluids.
2) Antibodies directed against LA were detected in serum samples taken before the piglets had sucked, or were detected in ascitic fluids of piglets born dead.

Table 7.
Reactivity in IPMA of a collection of field sera from Europe and North-America tested with LA isolates from the Netherlands (NL1 and NL2), Germany (GE1 and GE2), and the United States (US1, US2 and US3).

Isolates:	NL1	NL2	GE1	GE2	US1	US2	US3
Sera from:							
The Netherlands							
TH-187	3.5_t	3.5	2.5	3.5	-	-	-
TO-36	3.5	3.0	2.5	3.0	-	1.0	-
Germany							
BE-352	4.0	3.5	2.5	3.0	-	1.5	-
BE-392	3.5	3.5	2.5	2.5	1.5	1.5	0.5
NI-f2	2.5	1.5	2.0	2.5	-	-	-
United Kingdom							
PA-141615	4.0	3.0	3.0	3.5	-	-	-
PA-141617	4.0	3.5	3.0	3.5	-	2.5	2.0
PA-142440	3.5	3.0	2.5	3.5	-	2.0	2.5
Belgium							
PE-1960	4.5	4.5	3.0	4.0	1.5	-	-
France							
EA-2975	4.0	3.5	3.0	3.0	2.0	-	-
EA-2985	3.5	3.0	3.0	2.5	-	-	-
United States							
SL-441	3.5	1.5	2.5	2.5	3.5	3.5	3.0
SL-451	3.0	2.0	2.5	2.5	3.5	4.5	4.0
AL-RP9577	1.5	-	-	1.0	3.0	4.0	2.5
AL-P10814/33	0.5	2.5	-	-	2.5	3.5	3.0
AL-4094A	-	-	-	-	1.0	2.0	0.5
AL-7525	-	-	-	-	-	1.0	-
JC-MN41	-	-	-	-	1.0	3.5	1.0
JC-MN44	-	-	-	-	2.0	3.5	2.0
JC-MN45	-	-	-	-	2.0	3.5	2.5
Canada							
RB-16	2.5	-	3.0	2.0	3.0	3.5	-
RB-19	1.0	-	1.0	-	2.5	1.5	-
RB-22	1.5	-	2.0	2.5	2.5	3.5	-
RB-23	-	-	-	-	-	3.0	-

t = titre expressed as negative log; - = negative

Table 8.
Reactivity in IPMA of a collection of experimental sera raised against LA and SIRSV tested with LA isolates from the Netherlands (NL1 and NL2), Germany (GE1 and GE2), and the United States (US1, US2 and US3).

Isolates:		NL1	NL2	GE1	GE2	US1	US2	US3
Sera:								
anti-LA:								
21	14 dpi	2.5[t]	2.0	2.5	3.0	1.5	2.0	1.5
	28 dpi	4.0	3.5	3.5	4.0	-	2.5	1.5
	42 dpi	4.0	3.5	3.0	3.5	1.5	2.5	2.0
23	14 dpi	3.0	2.0	2.5	3.0	1.0	2.0	1.0
	28 dpi	3.5	3.5	3.5	4.0	1.5	2.0	2.0
	42 dpi	4.0	4.0	3.0	4.0	-	2.5	2.5
25	14 dpi	2.5	2.0	2.5	3.0	1.5	2.0	1.0
	28 dpi	4.0	3.5	4.0	3.5	-	1.5	2.0
	42 dpi	3.5	4.0	3.5	3.5	1.5	2.0	2.0
29	14 dpi	3.5	3.5	3.0	3.5	-	2.0	1.5
	28 dpi	3.5	3.5	3.0	3.5	-	2.5	2.0
	42 dpi	4.0	3.5	3.5	4.0	1.5	2.5	2.5
anti-SISRV:								
2B	20 dpi	-	-	-	-	2.0	2.0	-
	36 dpi	-	-	-	-	1.5	2.0	-
	63 dpi	-	-	-	-	1.0	1.0	-
9G	30 dpi	-	-	-	-	2.5	3.0	-
	44 dpi	-	-	-	-	2.5	3.5	-
	68 dpi	-	-	-	-	2.0	3.5	1.5
16W	25 dpi	-	-	-	-	2.0	3.0	-
	40 dpi	-	-	-	-	2.0	3.0	-
	64 dpi	-	-	-	-	2.5	2.5	1.5
16Y	36 dpi	-	-	-	-	1.0	3.0	1.0
	64 dpi	-	-	-	-	2.5	3.0	-

t = titer expressed as negative log; - = negative

Table 9.
Characteristics of the ORFs of Lelystad Virus.

ORF	Nucleotides (first-last)	No. of amino acids	Calculated size of the unmodified peptide (kDa)	number of glycosylation sites
ORF1A	212-7399	2396	260.0	3
ORF1B	7384-11772	1463	161.8	3
ORF2	11786-12532	249	28.4	2
ORF3	12394-13188	265	30.6	7
	12556-13188	211	24.5	4
ORF4	12936-13484	183	20.0	4
	12981-13484	168	18.4	4
	13068-13484	139	15.4	3
ORF5	13484-14086	201	22.4	2
ORF6	14077-14595	173	18.9	2
ORF7	14588-14971	128	13.8	1

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CLAIMS

1. Composition of matter comprising isolated Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

2. Composition of matter comprising killed isolated Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

3. Composition of matter comprising attenuated isolated Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

4. Composition of matter comprising a recombinant vector derived from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

5. Composition of matter comprising an isolated part or component of Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

6. Composition of matter comprising isolated or synthetic protein, (poly)peptide, or nucleic acid derived from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the

isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

7. Composition of matter comprising recombinant nucleic acid which comprises a nucleotide sequence derived from the genome of Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

8. Composition of matter comprising recombinant nucleic acid which comprises a Lelystad Agent-specific nucleotide sequence shown in figure 1.

9. Composition of matter comprising recombinant nucleic acid which comprises a Lelystad Agent-specific nucleotide sequence selected from anyone of the Open Reading Frames shown in figure 1.

10. Composition of matter comprising a (poly)peptide having an amino acid sequence derived from a protein of Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, the (poly)peptide being produced by a cell capable of producing it due to genetic engineering with appropriate recombinant DNA.

11. Composition of matter comprising a (poly)peptide comprising a Lelystad Agent-specific amino acid sequence shown in figure 1.

12. Composition of matter comprising an isolated or synthetic antibody which specifically recognizes a part or component of Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

13. Composition of matter comprising a recombinant vector which contains nucleic acid comprising a nucleotide sequence coding for a protein or antigenic peptide derived from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102.

14. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

15. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising killed Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

16. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising attenuated Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

17. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising a

recombinant vector which contains nucleic acid comprising a nucleotide sequence coding for a protein or antigenic peptide derived from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

18. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising an antigenic part or component of Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

19. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against Mystery Swine Disease, comprising a protein or antigenic polypeptide derived from, or a peptide mimicking an antigenic component of, Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, and a suitable carrier or adjuvant.

20. Vaccine composition for vaccinating animals, in particular mammals, more in particular pigs or swines, to protect them against a disease caused by a pathogen, comprising a recombinant vector derived from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, the nucleic acid of the recombinant vector comprising a nucleotide

sequence coding for a protein or antigenic peptide derived from the pathogen, and a suitable carrier or adjuvant.

21. Diagnostic kit for detecting nucleic acid from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising a nucleic acid probe or primer which comprises a nucleotide sequence derived from the genome of Lelystad Agent, and suitable detection means of a nucleic acid detection assay.

22. Diagnostic kit for detecting antigen from Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising an antibody which specifically recognizes a part or component of Lelystad Agent, and suitable detection means of an antigen detection assay.

23. Diagnostic kit for detecting an antibody which specifically recognizes Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising an antigenic part or component of Lelystad Agent, and suitable detection means of an antibody detection assay.

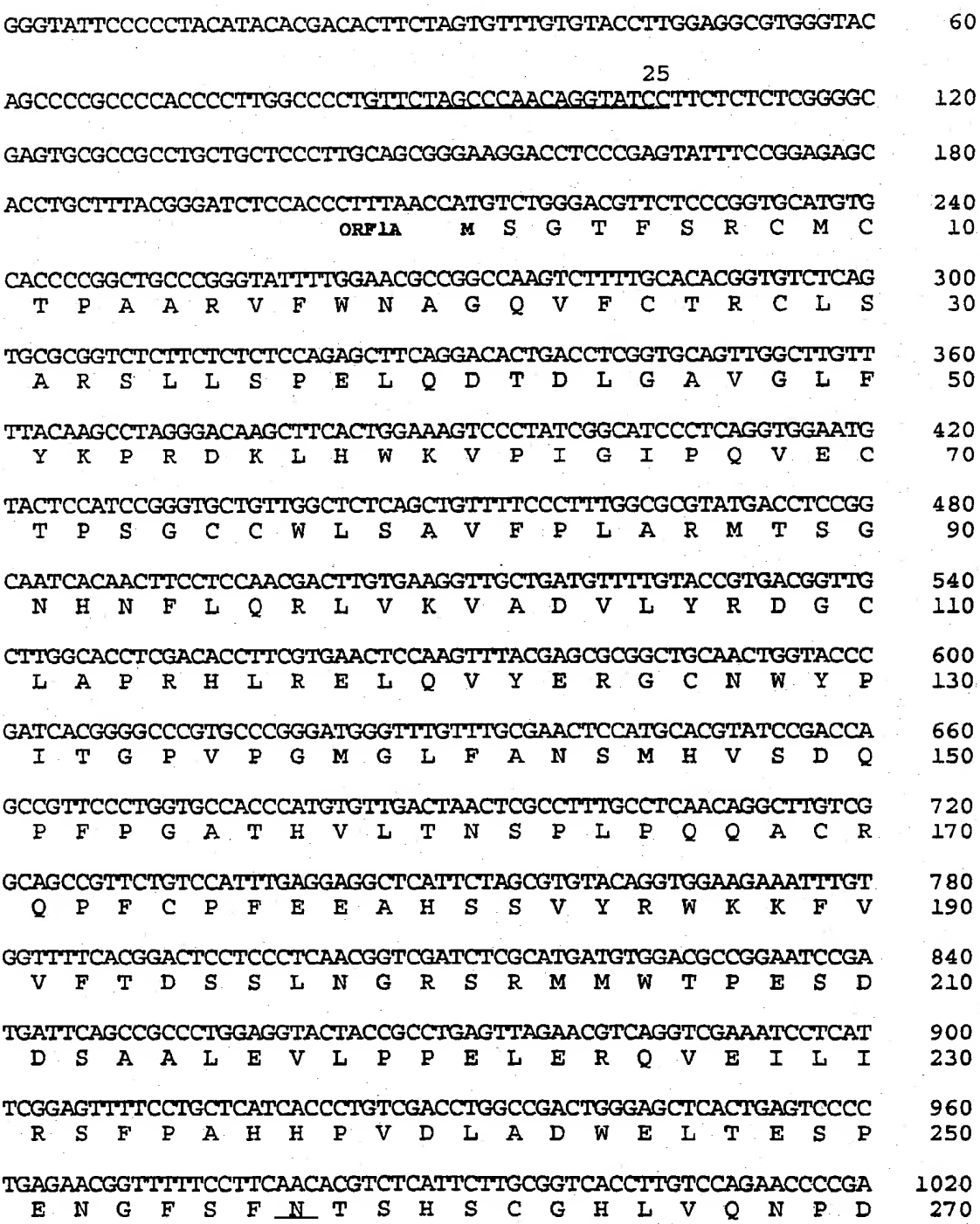
24. Diagnostic kit for detecting an antibody which specifically recognizes Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising a protein or antigenic polypeptide derived from Lelystad Agent, or a peptide mimicking an antigenic component of Lelystad Agent, and suitable detection means of an antibody detection assay.

25. Diagnostic kit for detecting an antibody which specifically recognizes Lelystad Agent which is the causative agent of Mystery Swine Disease, said Lelystad Agent essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991 with the Institut Pasteur, Paris, France, deposit number I-1102, in a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from an animal, in particular a mammal, more in particular a pig or swine, comprising killed, live or attenuated Lelystad Agent, and suitable detection means of an antibody detection assay.

26. A process for diagnosing whether an animal, in particular a mammal, more in particular a pig or swine, is contaminated with the causative agent of Mystery Swine Disease, comprising preparing a sample, in particular a biological sample such as blood or blood serum, sputum, saliva, or tissue, derived from the animal, and examining whether it contains Lelystad Agent nucleic acid, Lelystad Agent antigen, or antibody specifically recognizing Lelystad Agent, said Lelystad Agent being the causative agent of Mystery Swine Disease and essentially corresponding to the isolate Lelystad Agent (CDI-NL-2.91) deposited 5 June 1991

with the Institut Pasteur, Paris, France, deposit number I-1102.

Fig. 1(1)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25
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GAGTGCGCCGCCTGCTGCTCCCTTGCAGCGGGAAGGACCTCCCGAGTATTTCCGGAGAGC 180
ACCTGCTTTACGGGATCTCCACCCTTTAACCATGTCTGGGACGTTCTCCCGGTGCATGTG 240
ORF1A M S G T F S R C M C 10
CACCCCGGCTGCCCGGGTATTTTGGAACGCCGGCCAAGTCTTTTGCACACGGTGTCTCAG 300
T P A A R V F W N A G Q V F C T R C L S 30
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N H N F L Q R L V K V A D V L Y R D G C 110
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V F T D S S L N G R S R M M W T P E S D 210
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D S A A L E V L P P E L E R Q V E I L I 230
TCGGAGTTTTCCTGCTCATCACCCTGTCGACCTGGCCGACTGGGAGCTCACTGAGTCCCC 960
R S F P A H H P V D L A D W E L T E S P 250
TGAGAACGGTTTTTCCTTCAACACGTCTCATTCTTGCGGTCACCTTGTCCAGAACCCCGA 1020
E N G F S F N T S H S C G H L V Q N P D 270

Fig. 1(2)

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CGTGTTTGATGGCAAGTGCTGGCTCTCCTGCTTTTTGGGCCAGTCGGTCGAAGTGCGCTG   1080
  V  F  D  G  K  C  W  L  S  C  F  L  G  Q  S  V  E  V  R  C    290

CCATGAGGAACATCTAGCTGACGCCTTCGGTTACCAAACCAAGTGGGGCGTGCATGGTAA   1140
  H  E  E  H  L  A  D  A  F  G  Y  Q  T  K  W  G  V  H  G  K    310

GTACCTCCAGCGCAGGCTTCAAGTTCGCGGCATTCGTGCTGTAGTCGATCCTGATGGTCC   1200
  Y  L  Q  R  R  L  Q  V  R  G  I  R  A  V  V  D  P  D  G  P    330

CATTCACGTTGAAGCGCTGTCTTGCCCCCAGTCTTGGATCAGGCACCTGACTCTGGATGA   1260
  I  H  V  E  A  L  S  C  P  Q  S  W  I  R  H  L  T  L  D  D    350

TGATGTCACCCCAGGATTCGTTCGCCTGACATCCCTTCGCATTGTGCCGAACACAGAGCC   1320
  D  V  T  P  G  F  V  R  L  T  S  L  R  I  V  P  N  T  E  P    370

TACCACTTCCCGGATCTTTCGGTTTGGAGCGCATAAGTGGTATGGCGCTGCCGGCAAACG   1380
  T  T  S  R  I  F  R  F  G  A  H  K  W  Y  G  A  A  G  K  R    390

GGCTCGTGCTAAGCGTGCCGCTAAAAGTGAGAAGGATTCGGCTCCCACCCCCAAGGTTGC   1440
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CCTGCCGGTCCCCACCTGTGGAATTACCACCTACTCTCCACCGACAGACGGGTCTTGTGG   1500
  L  P  V  P  T  C  G  I  T  T  Y  S  P  P  T  D  G  S  C  G    430

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  W  H  V  L  A  A  I  M  N  R  M  I  N  G  D  F  T  S  P  L    450

GACTCAGTACAACAGACCAGAGGATGATTGGGCTTCTGATTATGATCTTGTTCAGGCGAT   1620
  T  Q  Y  N  R  P  E  D  D  W  A  S  D  Y  D  L  V  Q  A  I    470

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  Q  C  L  R  L  P  A  T  V  V  R  N  R  A  C  P  N  A  K  Y    490

CCTTATAAAACTTAACGGAGTTCACTGGGAGGTAGAGGTGAGGTCTGGAATGGCTCCTCG   1740
  L  I  K  L  N  G  V  H  W  E  V  E  V  R  S  G  M  A  P  R    510

CTCCCTTTCTCGTGAATGTGTGGTTGGCGTTTGCTCTGAAGGCTGTGTCGCACCGCCTTA   1800
  S  L  S  R  E  C  V  V  G  V  C  S  E  G  C  V  A  P  P  Y    530

TCCAGCAGACGGGCTACCTAAACGTGCACTCGAGGCCTTGGCGTCTGCTTACAGACTACC   1860
  P  A  D  G  L  P  K  R  A  L  E  A  L  A  S  A  Y  R  L  P    550

CTCCGATTGTGTTAGCTCTGGTATTGCTGACTTTCTTGCTAATCCACCTCCTCAGGAATT   1920
  S  D  C  V  S  S  G  I  A  D  F  L  A  N  P  P  P  Q  E  F    570

CTGGACCCTCGACAAAATGTTGACCTCCCCGTCACCAGAGCGGTCCGGCTTCTCTAGTTT   1980
  W  T  L  D  K  M  L  T  S  P  S  P  E  R  S  G  F  S  S  L    590
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Fig. 1(3)

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GTATAAATTACTATTAGAGGTTGTTCCGCAAAAATGCGGTGCCACGGAAGGGGCTTTCAT   2040
  Y  K  L  L  L  E  V  V  P  Q  K  C  G  A  T  E  G  A  F  I    610

CTATGCTGTTGAGAGGATGTTGAAGGATTGTCCGAGCTCCAAACAGGCCATGGCCCTTCT   2100
  Y  A  V  E  R  M  L  K  D  C  P  S  S  K  Q  A  M  A  L  L    630

GGCAAAAATTAAAGTTCCATCCTCAAAGGCCCCGTCTGTGTCCCTGGACGAGTGTTTCCC   2160
  A  K  I  K  V  P  S  S  K  A  P  S  V  S  L  D  E  C  F  P    650

TACGGATGTTTTAGCCGACTTCGAGCCAGCATCTCAGGAAAGGCCCCAAAGTTCCGGCGC   2220
  T  D  V  L  A  D  F  E  P  A  S  Q  E  R  P  Q  S  S  G  A    670

                                                  A
TGCTGTTGTCCTGTGTTCACCGGATGCAAAAGAGTTCGAGGAAGCAGCCCCGGAAGAAGT   2280
  A  V  V  L  C  S  P  D  A  K  E  F  E  E  A  A  P  E  E  V    690

TCAAGAGAGTGGCCACAAGGCCGTCCACTCTGCACTCCTTGCCGAGGGTCCTAACAATGA   2340
  Q  E  S  G  H  K  A  V  H  S  A  L  L  A  E  G  P  N  N  E    710

GCAGGTACAGGTGGTTGCCGGTGAGCAACTGAAGCTCGGCGGTTGTGGTTTGGCAGTCGG   2400
  Q  V  Q  V  V  A  G  E  Q  L  K  L  G  G  C  G  L  A  V  G    730

GAATGCTCATGAAGGTGCTCTGGTCTCAGCTGGTCTAATTAACCTGGTAGGCGGGAATTT   2460
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GTCCCCCTCAGACCCCATGAAAGAAAACATGCTCAATAGCCGGGAAGACGAACCACTGGA   2520
  S  P  S  D  P  M  K  E  N  M  L  N  S  R  E  D  E  P  L  D    770

TTTGTCCCAACCAGCACCAGCTTCCACAACGACCCTTGTGAGAGAGCAAACACCCGACAA   2580
  L  S  Q  P  A  P  A  S  T  T  T  L  V  R  E  Q  T  P  D  N    790

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TATACTCTGTCATGTTGAGCACTGCGGCACGGAGTCGGGCGACAGCAGTTCGCCTTTGGA   2700
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TCTATCTGATGCGCAAACCCTGGACCAGCCTTTAAATCTATCCCTGGCCGCTTGGCCAGT   2760
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GAGGGCCACCGCGTCTGACCCTGGCTGGGTCCACGGTAGGCGCGAGCCTGTCTTTGTAAA   2820
  R  A  T  A  S  D  P  G  W  V  H  G  R  R  E  P  V  F  V  K    870

GCCTCGAAATGCTTTCTCTGATGGCGATTCAGCCCTTCAGTTCGGGGAGCTTTCTGAATC   2880
  P  R  N  A  F  S  D  G  D  S  A  L  Q  F  G  E  L  S  E  S    890
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Fig. 1(4)

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CAGCTCTGTCATCGAGTTTGACCGGACAAAAGATGCTCCGGTGGTTGACGCCCCTGTCGA   2940
  S  S  V  I  E  F  D  R  T  K  D  A  P  V  V  D  A  P  V  D    910

CTTGACGACTTCGAACGAGGCCCTCTCTGTAGTCGATCCTTTCGAATTTGCCGAACTCAA   3000
  L  T  T  S  N  E  A  L  S  V  V  D  P  F  E  F  A  E  L  K    930

GCGCCCGCGTTTCTCCGCACAAGCCTTAATTGACCGAGGCGGTCCACTTGCCGATGTCCA   3060
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TGCAACCCCAGCCACCAGGGAGTGGCTCGACAAAATGTGGGATAGGGTGGACATGAAAAC   3180
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  G  L  K  Q  L  V  A  Q  W  D  R  K  L  S  V  T  P  P  P  K   1050

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CCTTATGACATGGGTTTTTGAAGTTTTCTCCCACCTCCCAGCTTTTATGCTCACACTTTT   3600
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CTCGCCGCGGGGCTCTATGGCTCCAGGTGATTGGTTGTTTGCAGGTGTCGTTTTACTTGC   3660
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TCTCTTGCTCTGTCGTTCTTACCCGATACTCGGATGCCTTCCCTTATTGGGTGTCTTTTC   3720
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TGGTTCTTTGCGGCGTGTTCGTCTGGGTGTTTTTGGTTCTTGGATGGCTTTTGCTGTATT   3780
  G  S  L  R  R  V  R  L  G  V  F  G  S  W  M  A  F  A  V  F   1190

TTTATTCTCGACTCCATCCAACCCAGTCGGTTCTTCTTGTGACCACGATTCGCCGGAGTG   3840
  L  F  S  T  P  S  N  P  V  G  S  S  C  D  H  D  S  P  E  C   1210
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Fig. 1(5)

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TCATGCTGAGCTTTTGGCTCTTGAGCAGCGCCAACTTTGGGAACCTGTGCGCGGCCTTGT   3900
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GGTCGGCCCCTCAGGCCTCTTATGTGTCATTCTTGGCAAGTTACTCGGTGGGTCACGTTA   3960
  V  G  P  S  G  L  L  C  V  I  L  G  K  L  L  G  G  S  R  Y   1250

TCTCTGGCATGTTCTCCTACGTTTATGCATGCTTGCAGATTTGGCCCTTTCTCTTGTTTA   4020
  L  W  H  V  L  L  R  L  C  M  L  A  D  L  A  L  S  L  V  Y   1270

TGTGGTGTCCCAGGGGCGTTGTCACAAGTGTTGGGGAAAGTGTATAAGGACAGCTCCTGC   4080
  V  V  S  Q  G  R  C  H  K  C  W  G  K  C  I  R  T  A  P  A   1290

GGAGGTGGCTCTTAATGTATTTCCTTTCTCGCGCGCCACCCGTGTCTCTCTTGTATCCTT   4140
  E  V  A  L  N  V  F  P  F  S  R  A  T  R  V  S  L  V  S  L   1310

GTGTGATCGATTCCAAACGCCAAAAGGGGTTGATCCTGTGCACTTGGCAACGGGTTGGCG   4200
  C  D  R  F  Q  T  P  K  G  V  D  P  V  H  L  A  T  G  W  R   1330

CGGGTGCTGGCGTGGTGAGAGCCCCATCCATCAACCACACCAAAAGCCCATAGCTTATGC   4260
  G  C  W  R  G  E  S  P  I  H  Q  P  H  Q  K  P  I  A  Y  A   1350

CAATTTGGATGAAAAGAAAATGTCTGCCCAAACGGTGGTTGCTGTCCCATACGATCCCAG   4320
  N  L  D  E  K  K  M  S  A  Q  T  V  V  A  V  P  Y  D  P  S   1370

TCAGGCTATCAAATGCCTGAAAGTTCTGCAGGCGGGAGGGGCCATCGTGGACCAGCCTAC   4380
  Q  A  I  K  C  L  K  V  L  Q  A  G  G  A  I  V  D  Q  P  T   1390

ACCTGAGGTCGTTCGTGTGTCCGAGATCCCCTTCTCAGCCCCATTTTTCCCAAAAGTTCC   4440
  P  E  V  V  R  V  S  E  I  P  F  S  A  P  F  F  P  K  V  P   1410

AGTCAACCCAGATTGCAGGGTTGTGGTAGATTCGGACACTTTTGTGGCTGCGGTTCGCTG   4500
  V  N  P  D  C  R  V  V  V  D  S  D  T  F  V  A  A  V  R  C   1430

                        C
CGGTTACTCGACAGCACAACTGGTTCTGGGCCGGGGCAACTTTGCCAAGTTAAATCAGAC   4560
  G  Y  S  T  A  Q  L  V  L  G  R  G  N  F  A  K  L  N  Q  T   1450

CCCCCCCAGGAACTCTATCTCCACCAAAACGACTGGTGGGGCCTCTTACACCCTTGCTGT   4620
  P  P  R  N  S  I  S  T  K  T  T  G  G  A  S  Y  T  L  A  V   1470

GGCTCAAGTGTCTGCGTGGACTCTTGTTCATTTCATCCTCGGTCTTTGGTTCACATCACC   4680
  A  Q  V  S  A  W  T  L  V  H  F  I  L  G  L  W  F  T  S  P   1490

TCAAGTGTGTGGCCGAGGAACCGCTGACCCATGGTGTTCAAATCCTTTTTCATATCCTAC   4740
  Q  V  C  G  R  G  T  A  D  P  W  C  S  N  P  F  S  Y  P  T   1510

CTATGGCCCCGGAGTTGTGTGCTCCTCTCGACTTTGTGTGTCTGCCGACGGGGTCACCCT   4800
  Y  G  P  G  V  V  C  S  S  R  L  C  V  S  A  D  G  V  T  L   1530
```

Fig. 1(6)

```
GCCATTGTTCTCAGCCGTGGCACAACTCTCCGGTAGAGAGGTGGGGATTTTTATTTTGGT   4860
  P  L  F  S  A  V  A  Q  L  S  G  R  E  V  G  I  F  I  L  V   1550

GCTCGTCTCCTTGACTGCTTTGGCCCACCGCATGGCTCTTAAGGCAGACATGTTAGTGGT   4920
  L  V  S  L  T  A  L  A  H  R  M  A  L  K  A  D  M  L  V  V   1570

CTTTTCGGCTTTTTGTGCTTACGCCTGGCCCATGAGCTCCTGGTTAATCTGCTTCTTTCC   4980
  F  S  A  F  C  A  Y  A  W  P  M  S  S  W  L  I  C  F  F  P   1590

TATACTCTTGAAGTGGGTTACCCTTCACCCTCTTACTATGCTTTGGGTGCACTCATTCTT   5040
  I  L  L  K  W  V  T  L  H  P  L  T  M  L  W  V  H  S  F  L   1610

GGTGTTTTGTCTGCCAGCAGCCGGCATCCTCTCACTAGGGATAACTGGCCTTCTTTGGGC   5100
  V  F  C  L  P  A  A  G  I  L  S  L  G  I  T  G  L  L  W  A   1630

AATTGGCCGCTTTACCCAGGTTGCCGGAATTATTACACCTTATGACATCCACCAGTACAC   5160
  I  G  R  F  T  Q  V  A  G  I  I  T  P  Y  D  I  H  Q  Y  T   1650

CTCTGGGCCACGTGGTGCAGCTGCTGTGGCCACAGCCCCAGAAGGCACTTATATGGCCGC   5220
  S  G  P  R  G  A  A  A  V  A  T  A  P  E  G  T  Y  M  A  A   1670

CGTCCGGAGAGCTGCTTTAACTGGGCGAACTTTAATCTTCACCCCGTCTGCAGTTGGATC   5280
  V  R  R  A  A  L  T  G  R  T  L  I  F  T  P  S  A  V  G  S   1690

CCTTCTCGAAGGTGCTTTCAGGACTCATAAACCCTGCCTTAACACCGTGAATGTTGTAGG   5340
  L  L  E  G  A  F  R  T  H  K  P  C  L  N  T  V  N  V  V  G   1710

CTCTTCCCTTGGTTCCGGAGGGGTTTTCACCATTGATGGCAGAAGAACTGTCGTCACTGC   5400
  S  S  L  G  S  G  G  V  F  T  I  D  G  R  R  T  V  V  T  A   1730

TGCCCATGTGTTGAACGGCGACACAGCTAGAGTCACCGGCGACTCCTACAACCGCATGCA   5460
  A  H  V  L  N  G  D  T  A  R  V  T  G  D  S  Y  N  R  M  H   1750

CACTTTCAAGACCAATGGTGATTATGCCTGGTCCCATGCTGATGACTGGCAGGGCGTTGC   5520
  T  F  K  T  N  G  D  Y  A  W  S  H  A  D  D  W  Q  G  V  A   1770

CCCTGTGGTCAAGGTTGCGAAGGGGTACCGCGGTCGTGCCTACTGGCAAACATCAACTGG   5580
  P  V  V  K  V  A  K  G  Y  R  G  R  A  Y  W  Q  T  S  T  G   1790

TGTCGAACCCGGTATCATTGGGGAAGGGTTCGCCTTCTGTTTTACTAACTGCGGCGATTC   5640
  V  E  P  G  I  I  G  E  G  F  A  F  C  F  T  N  C  G  D  S   1810

GGGGTCACCCGTCATCTCAGAATCTGGTGATCTTATTGGAATCCACACCGGTTCAAACAA   5700
  G  S  P  V  I  S  E  S  G  D  L  I  G  I  H  T  G  S  N  K   1830

ACTTGGTTCTGGTCTTGTGACAACCCCTGAAGGGGAGACCTGCACCATCAAAGAAACCAA   5760
  L  G  S  G  L  V  T  T  P  E  G  E  T  C  T  I  K  E  T  K   1850
```

Fig. 1(7)

```
GCTCTCTGACCTTTCCAGACATTTTGCAGGCCCAAGCGTTCCTCTTGGGGACATTAAATT   5820
  L  S  D  L  S  R  H  F  A  G  P  S  V  P  L  G  D  I  K  L   1870

GAGTCCGGCCATCATCCCTGATGTAACATCCATTCCGAGTGACTTGGCATCGCTCCTAGC   5880
  S  P  A  I  I  P  D  V  T  S  I  P  S  D  L  A  S  L  L  A   1890

CTCCGTCCCTGTAGTGGAAGGCGGCCTCTCGACCGTTCAACTTTTGTGTGTCTTTTTCCT   5940
  S  V  P  V  V  E  G  G  L  S  T  V  Q  L  L  C  V  F  F  L   1910

TCTCTGGCGCATGATGGGCCATGCCTGGACACCCATTGTTGCCGTGGGCTTCTTTTTGCT   6000
  L  W  R  M  M  G  H  A  W  T  P  I  V  A  V  G  F  F  L  L   1930

GAATGAAATTCTTCCAGCAGTTTTGGTCCGAGCCGTGTTTTCTTTTGCACTCTTTGTGCT   6060
  N  E  I  L  P  A  V  L  V  R  A  V  F  S  F  A  L  F  V  L   1950

TGCATGGGCCACCCCCTGGTCTGCACAGGTGTTGATGATTAGACTCCTCACGGCATCTCT   6120
  A  W  A  T  P  W  S  A  Q  V  L  M  I  R  L  L  T  A  S  L   1970

CAACCGCAACAAGCTTTCTCTGGCGTTCTACGCACTCGGGGGTGTCGTCGGTTTGGCAGC   6180
  N  R  N  K  L  S  L  A  F  Y  A  L  G  G  V  V  G  L  A  A   1990

TGAAATCGGGACTTTTGCTGGCAGATTGTCTGAATTGTCTCAAGCTCTTTCGACATACTG   6240
  E  I  G  T  F  A  G  R  L  S  E  L  S  Q  A  L  S  T  Y  C   2010

CTTCTTACCTAGGGTCCTTGCTATGACCAGTTGTGTTCCCACCATCATCATTGGTGGACT   6300
  F  L  P  R  V  L  A  M  T  S  C  V  P  T  I  I  I  G  G  L   2030

                        G
CCATACCCTCGGTGTGATTCTGTGGTTATTCAAATACCGGTGCCTCCACAACATGCTGGT   6360
  H  T  L  G  V  I  L  W  L  F  K  Y  R  C  L  H  N  M  L  V   2050

TGGTGATGGGAGTTTTTCAAGCGCCTTCTTCCTACGGTATTTTGCAGAGGGTAATCTCAG   6420
  G  D  G  S  F  S  S  A  F  F  L  R  Y  F  A  E  G  N  L  R   2070

AAAAGGTGTTTCACAGTCCTGTGGCATGAATAACGAGTCCCTAACGGCTGCTTTAGCTTG   6480
  K  G  V  S  Q  S  C  G  M  N  N  E  S  L  T  A  A  L  A  C   2090

CAAGTTGTCACAGGCTGACCTTGATTTTTTGTCCAGCTTAACGAACTTCAAGTGCTTTGT   6540
  K  L  S  Q  A  D  L  D  F  L  S  S  L  T  N  F  K  C  F  V   2110

ATCTGCTTCAAACATGAAAAATGCTGCCGGCCAGTACATTGAAGCAGCGTATGCCAAGGC   6600
  S  A  S  N  M  K  N  A  A  G  Q  Y  I  E  A  A  Y  A  K  A   2130

CCTGCGCCAAGAGTTGGCCTCTCTAGTTCAGATTGACAAAATGAAAGGAGTTTTGTCCAA   6660
  L  R  Q  E  L  A  S  L  V  Q  I  D  K  M  K  G  V  L  S  K   2150
```

Fig. 1(8)

```
GCTCGAGGCCTTTGCTGAAACAGCCACCCCGTCCCTTGACATAGGTGACGTGATTGTTCT   6720
  L  E  A  F  A  E  T  A  T  P  S  L  D  I  G  D  V  I  V  L   2170

GCTTGGGCAACATCCTCACGGATCCATCCTCGATATTAATGTGGGGACTGAAAGGAAAAC   6780
  L  G  Q  H  P  H  G  S  I  L  D  I  N  V  G  T  E  R  K  T   2190

TGTGTCCGTGCAAGAGACCCGGAGCCTAGGCGGCTCCAAATTCAGTGTTTGTACTGTCGT   6840
  V  S  V  Q  E  T  R  S  L  G  G  S  K  F  S  V  C  T  V  V   2210

                          A
GTCCAACACACCCGTGGACGCCTTGACCGGCATCCCACTCCAGACACCAACCCCTCTTTT   6900
  S  N  T  P  V  D  A  L  T  G  I  P  L  Q  T  P  T  P  L  F   2230

TGAGAATGGTCCGCGTCATCGCAGCGAGGAAGACGATCTTAAAGTCGAGAGGATGAAGAA   6960
  E  N  G  P  R  H  R  S  E  E  D  D  L  K  V  E  R  M  K  K   2250

ACACTGTGTATCCCTCGGCTTCCACAACATCAATGGCAAAGTTTACTGCAAAATTTGGGA   7020
  H  C  V  S  L  G  F  H  N  I  N  G  K  V  Y  C  K  I  W  D   2270

CAAGTCTACCGGTGACACCTTTTACACGGATGATTCCCGGTACACCCAAGACCATGCTTT   7080
  K  S  T  G  D  T  F  Y  T  D  D  S  R  Y  T  Q  D  H  A  F   2290

TCAGGACAGGTCAGCCGACTACAGAGACAGGGACTATGAGGGTGTGCAAACCACCCCCCA   7140
  Q  D  R  S  A  D  Y  R  D  R  D  S  E  T  P  V  G  T  V  V   2310

ACAGGGATTTGATCCAAAGTCTGAAACCCCTGTTGGCACTGTTGTGATCGGCGGTATTAC   7200
  I  G  G  I  T  Y  Y  E  G  V  Q  T  T  P  Q  Q  G  F  D  P   2330

GTATAACAGGTATCTGATCAAAGGTAAGGAGGTTCTGGTCCCCAAGCCTGACAACTGCCT   7260
  K  N  R  Y  L  I  K  G  K  E  V  L  V  P  K  P  D  N  C  L   2350

TGAAGCTGCCAAGCTGTCCCTTGAGCAAGCTCTCGCTGGGATGGGCCAAACTTGCGACCT   7320
  E  A  A  K  L  S  L  E  Q  A  L  A  G  M  G  Q  T  C  D  L   2370

TACAGCTGCCGAGGTGGAAAAGCTAAAGCGCATCATTAGTCAACTCCAAGGTTTGACCAC   7380
  T  A  A  E  V  E  K  L  K  R  I  I  S  Q  L  Q  G  L  T  T   2390
                                                    ORF1B

TGAACAGGCTTTAAACTGTTAGCCGCCAGCGGCTTGACCCGCTGTGGCCGCGGCGGCCTA   7440
  E  Q  A  L  N  C  -                                           2396
  -  T  G  F  K  L  L  A  A  S  G  L  T  R  C  G  R  G  G  L    19

GTTGTGACTGAAACGGCGGTAAAAATTATAAAATACCACAGCAGAACTTTCACCTTAGGC   7500
  V  V  T  E  T  A  V  K  I  I  K  Y  H  S  R  T  F  T  L  G    39

CCTTTAGACCTAAAAGTCACTTCCGAGGTGGAGGTAAAGAAATCAACTGAGCAGGGCCAC   7560
  P  L  D  L  K  V  T  S  E  V  E  V  K  K  S  T  E  Q  G  H    59
```

Fig. 1(9)

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GCTGTTGTGGCAAACTTATGTTCCGGTGTCATCTTGATGAGACCTCACCCACCGTCCCTT   7620
 A  V  V  A  N  L  C  S  G  V  I  L  M  R  P  H  P  P  S  L     79

GTCGACGTTCTTCTGAAACCCGGACTTGACACAATACCCGGCATTCAACCAGGGCATGGG   7680
 V  D  V  L  L  K  P  G  L  D  T  I  P  G  I  Q  P  G  H  G     99

GCCGGGAATATGGGCGTGGACGGTTCTATTTGGGATTTTGAAACCGCACCCACAAAGGCA   7740
 A  G  N  M  G  V  D  G  S  I  W  D  F  E  T  A  P  T  K  A    119

GAACTCGAGTTATCCAAGCAAATAATCCAAGCATGTGAAGTTAGGCGCGGGGACGCCCCG   7800
 E  L  E  L  S  K  Q  I  I  Q  A  C  E  V  R  R  G  D  A  P    139

AACCTCCAACTCCCTTACAAGCTCTATCCTGTTAGGGGGGATCCTGAGCGGCATAAAGGC   7860
 N  L  Q  L  P  Y  K  L  Y  P  V  R  G  D  P  E  R  H  K  G    159

CGCCTTATCAATACCAGGTTTGGAGATTTACCTTACAAAACTCCTCAAGACACCAAGTCC   7920
 R  L  I  N  T  R  F  G  D  L  P  Y  K  T  P  Q  D  T  K  S    179

GCAATCCACGCGGCTTGTTGCCTGCACCCCAACGGGGCCCCCGTGTCTGATGGTAAATCC   7980
 A  I  H  A  A  C  C  L  H  P  N  G  A  P  V  S  D  G  K  S    199

ACACTAGGTACCACTCTTCAACATGGTTTCGAGCTTTATGTCCCTACTGTGCCCTATAGT   8040
 T  L  G  T  T  L  Q  H  G  F  E  L  Y  V  P  T  V  P  Y  S    219

GTCATGGAGTACCTTGATTCACGCCCTGACACCCCTTTTATGTGTACTAAACATGGCACT   8100
 V  M  E  Y  L  D  S  R  P  D  T  P  F  M  C  T  K  H  G  T    239

TCCAAGGCTGCTGCAGAGGACCTCCAAAAATACGACCTATCCACCCAAGGATTTGTCCTG   8160
 S  K  F  V  L  P  G  V  L  R  L  V  R  R  F  I  F  A  A  A    259

CCTGGGGTCCTACGCCTAGTACGCAGATTCATCTTTGGCCATATTGGTAAGGCGCCGCCA   8220
 E  D  L  Q  K  Y  D  L  S  T  Q  G  G  H  I  G  K  A  P  P    279

TTGTTCCTCCCATCAACCTATCCCGCCAAGAACTCTATGGCAGGGATCAATGGCCAGAGG   8280
 L  F  L  P  S  T  Y  P  A  K  N  S  M  A  G  I  N  G  Q  R    299

TTCCCAACAAAGGACGTTCAGAGCATACCTGAAATTGATGAAATGTGTGCCCGCGCTGTC   8340
 F  P  T  K  D  V  Q  S  I  P  E  I  D  E  M  C  A  R  A  V    319

AAGGAGAATTGGCAAACTGTGACACCTTGCACCCTCAAGAAACAGTACTGTTCCAAGCCC   8400
 K  E  N  W  Q  T  V  T  P  C  T  L  K  K  Q  Y  C  S  K  P    339

AAAACCAGGACCATCCTGGGCACCAACAACTTTATTGCCTTGGCTCACAGATCGGCGCTC   8460
 K  T  R  T  I  L  G  T  N  N  F  I  A  L  A  H  R  S  A  L    359

AGTGGTGTCACCCAGGCATTCATGAAGAAGGCTTGGAAGTCCCCAATTGCCTTGGGGAAA   8520
 S  G  V  T  Q  A  F  M  K  K  A  W  K  S  P  I  A  L  G  K    379
```

Fig. 1(10)

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AACAAATTCAAGGAGCTGCATTGCACTGTCGCCGGCAGGTGTCTTGAGGCCGACTTGGCC   8580
 N  K  F  K  E  L  H  C  T  V  A  G  R  C  L  E  A  D  L  A     399

TCCTGTGACCGCAGCACCCCCGCCATTGTAAGATGGTTTGTTGCCAACCTCCTGTATGAA   8640
 S  C  D  R  S  T  P  A  I  V  R  W  F  V  A  N  L  L  Y  E     419

CTTGCAGGATGTGAAGAGTACTTGCCTAGCTATGTGCTTAATTGCTGCCATGACCTCGTG   8700
 L  A  G  C  E  E  Y  L  P  S  Y  V  L  N  C  C  H  D  L  V     439

GCAACACAGGATGGTGCCTTCACAAAACGCGGTGGCCTGTCGTCCGGGGACCCCGTCACC   8760
 A  T  Q  D  G  A  F  T  K  R  G  G  L  S  S  G  D  P  V  T     459

AGTGTGTCCAACACCGTATATTCACTGGTAATTTATGCCCAGCACATGGTATTGTCGGCC   8820
 S  V  S  N  T  V  Y  S  L  V  I  Y  A  Q  H  M  V  L  S  A     479

TTGAAAATGGGTCATGAAATTGGTCTTAAGTTCCTCGAGGAACAGCTCAAGTTCGAGGAC   8880
 L  K  M  G  H  E  I  G  L  K  F  L  E  E  Q  L  K  F  E  D     499

CTCCTTGAAATTCAGCCTATGTTGGTATACTCTGATGATCTTGTCTTGTACGCTGAAAGA   8940
 L  L  E  I  Q  P  M  L  V  Y  S  D  D  L  V  L  Y  A  E  R     519

    C
CCCACATTTCCCAATTACCACTGGTGGGTCGAGCACCTTGACCTGATGCTGGGTTTCAGA   9000
 P  T  F  P  N  Y  H  W  W  V  E  H  L  D  L  M  L  G  F  R     539

ACGGACCCAAAGAAAACCGTCATAACTGATAAACCCAGCTTCCTCGGCTGCAGAATTGAG   9060
 T  D  P  K  K  T  V  I  T  D  K  P  S  F  L  G  C  R  I  E     559

GCAGGGCGACAGCTAGTCCCCAATCGCGACCGCATCCTGGCTGCTCTTGCATATCACATG   9120
 A  G  R  Q  L  V  P  N  R  D  R  I  L  A  A  L  A  Y  H  M     579

AAGGCGCAGAACGCCTCAGAGTATTATGCGTCTGCTGCCGCAATCCTGATGGATTCATGT   9180
 K  A  Q  N  A  S  E  Y  Y  A  S  A  A  A  I  L  M  D  S  C     599

GCTTGCATTGACCATGACCCTGAGTGGTATGAGGACCTCATCTGCGGTATTGCCCGGTGC   9240
 A  C  I  D  H  D  P  E  W  Y  E  D  L  I  C  G  I  A  R  C     619

GCCCGCCAGGATGGTTATAGCTTCCCAGGTCCGGCATTTTTCATGTCCATGTGGGAGAAG   9300
 A  R  Q  D  G  Y  S  F  P  G  P  A  F  F  M  S  M  W  E  K     639

CTGAGAAGTCATAATGAAGGGAAGAAATTCCGCCACTGCGGCATCTGCGACGCCAAAGCC   9360
 L  R  S  H  N  E  G  K  K  F  R  H  C  G  I  C  D  A  K  A     659

GACTATGCGTCCGCCTGTGGGCTTGATTTGTGTTTGTTCCATTCGCACTTTCATCAACAC   9420
 D  Y  A  S  A  C  G  L  D  L  C  L  F  H  S  H  F  H  Q  H     679
```

Fig. 1(11)

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     C
TGCCCTGTCACTCTGAGCTGCGGTCACCATGCCGGTTCAAAGGAATGTTCGCAGTGTCAG   9480
 C  P  V  T  L  S  C  G  H  H  A  G  S  K  E  C  S  Q  C  Q     699

TCACCTGTTGGGGCTGGCAGATCCCCTCTTGATGCCGTGCTAAAACAAATTCCATACAAA   9540
 S  P  V  G  A  G  R  S  P  L  D  A  V  L  K  Q  I  P  Y  K     719

CCTCCTCGTACTGTCATCATGAAGGTGGGTAATAAAACAACGGCCCTCGATCCGGGGAGG   9600
 P  P  R  T  V  I  M  K  V  G  N  K  T  T  A  L  D  P  G  R     739

TACCAGTCCCGTCGAGGTCTCGTTGCAGTCAAGAGGGGTATTGCAGGCAATGAAGTTGAT   9660
 Y  Q  S  R  R  G  L  V  A  V  K  R  G  I  A  G  N  E  V  D     759

        A
CTTTCTGATGGGGACTACCAAGTGGTGCCTCTTTTGCCGACTTGCAAAGACATAAACATG   9720
 L  S  D  G  D  Y  Q  V  V  P  L  L  P  T  C  K  D  I  N  M     779

GTGAAGGTGGCTTGCAATGTACTACTCAGCAAGTTCATAGTAGGGCCACCAGGTTCCGGA   9780
 V  K  V  A  C  N  V  L  L  S  K  F  I  V  G  P  P  G  S  G     799

                                                T
AAGACCACCTGGCTACTGAGTCAAGTCCAGGACGATGATGTCATTTACACACCCACCCAT   9840
 K  T  T  W  L  L  S  Q  V  Q  D  D  D  V  I  Y  T  P  T  H     819
                                                   I

CAGACTATGTTTGATATAGTCAGTGCTCTCAAAGTTTGCAGGTATTCCATTCCAGGAGCC   9900
 Q  T  M  F  D  I  V  S  A  L  K  V  C  R  Y  S  I  P  G  A     839

TCAGGACTCCCTTTCCCACCACCTGCCAGGTCCGGGCCGTGGGTTAGGCTTATTGCCAGC   9960
 S  G  L  P  F  P  P  P  A  R  S  G  P  W  V  R  L  I  A  S     859

GGGCACGTCCCTGGCCGAGTATCATACCTCGATGAGGCTGGATATTGTAATCATCTGGAC  10020
 G  H  V  P  G  R  V  S  Y  L  D  E  A  G  Y  C  N  H  L  D     879

ATTCTTAGACTGCTTTCCAAAACACCCCTTGTGTGTTTGGGTGACCTTCAGCAACTTCAC  10080
 I  L  R  L  L  S  K  T  P  L  V  C  L  G  D  L  Q  Q  L  H     899

CCTGTCGGCTTTGATTCCTACTGTTATGTGTTCGATCAGATGCCTCAGAAGCAGCTGACC  10140
 P  V  G  F  D  S  Y  C  Y  V  F  D  Q  M  P  Q  K  Q  L  T     919

ACTATTTACAGATTTGGCCCTAACATCTGCGCACGCATCCAGCCTTGTTACAGGGAGAAA  10200
 T  I  Y  R  F  G  P  N  I  C  A  R  I  Q  P  C  Y  R  E  K     939

CTTGAATCTAAGGCTAGGAACACTAGGGTGGTTTTTACCACCCGGCCTGTGGCCTTTGGT  10260
 L  E  S  K  A  R  N  T  R  V  V  F  T  T  R  P  V  A  F  G     959

CAGGTGCTGACACCATACCATAAAGATCGCATCGGCTCTGCGATAACCATAGATTCATCC  10320
 Q  V  L  T  P  Y  H  K  D  R  I  G  S  A  I  T  I  D  S  S     979
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Fig. 1(12)

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CAGGGGGCCACCTTTGATATTGTGACATTGCATCTACCATCGCCAAAGTCCCTAAATAAA  10380
 Q  G  A  T  F  D  I  V  T  L  H  L  P  S  P  K  S  L  N  K    999

TCCCGAGCACTTGTAGCCATCACTCGGGCAAGACACGGGTTGTTCATTTATGACCCTCAT  10440
 S  R  A  L  V  A  I  T  R  A  R  H  G  L  F  I  Y  D  P  H   1019

AACCAGCTCCAGGAGTTTTTCAACTTAACCCCTGAGCGCACTGATTGTAACCTTGTGTTC  10500
 N  Q  L  Q  E  F  F  N  L  T  P  E  R  T  D  C  N  L  V  F   1039

AGCCGTGGGGATGAGCTGGTAGTTCTGAATGCGGATAATGCAGTCACAACTGTAGCGAAG  10560
 S  R  G  D  E  L  V  V  L  N  A  D  N  A  V  T  T  V  A  K   1059

GCCCTTGAGACAGGTCCATCTCGATTTCGAGTATCAGACCCGAGGTGCAAGTCTCTCTTA  10620
 A  L  E  T  G  P  S  R  F  R  V  S  D  P  R  C  K  S  L  L   1079

GCCGCTTGTTCGGCCAGTCTGGAAGGGAGCTGTATGCCACTACCGCAAGTGGCACATAAC  10680
 A  A  C  S  A  S  L  E  G  S  C  M  P  L  P  Q  V  A  H  N   1099

CTGGGGTTTTACTTTTCCCCGGACAGTCCAACATTTGCACCTCTGCCAAAAGAGTTGGCG  10740
 L  G  F  Y  F  S  P  D  S  P  T  F  A  P  L  P  K  E  L  A   1119

CCACATTGGCCAGTGGTTACCCACCAGAATAATCGGGCGTGGCCTGATCGACTTGTCGCT  10800
 P  H  W  P  V  V  T  H  Q  N  N  R  A  W  P  D  R  L  V  A   1139

AGTATGCGCCCAATTGATGCCCGCTACAGCAAGCCAATGGTCGGTGCAGGGTATGTGGTC  10860
 S  M  R  P  I  D  A  R  Y  S  K  P  M  V  G  A  G  Y  V  V   1159

GGGCCGTCCACCTTTCTTGGTACTCCTGGTGTGGTGTCATACTATCTCACACTATACATC  10920
 G  P  S  T  F  L  G  T  P  G  V  V  S  Y  Y  L  T  L  Y  I   1179

AGGGGTGAGCCCCAGGCCTTGCCAGAAACACTCGTTTCAACAGGGCGTATAGCCACAGAT  10980
 R  G  E  P  Q  A  L  P  E  T  L  V  S  T  G  R  I  A  T  D   1199

TGTCGGGAGTATCTCGACGCGGCTGAGGAAGAGGCAGCAAAAGAACTCCCCCACGCATTC  11040
 C  R  E  Y  L  D  A  A  E  E  E  A  A  K  E  L  P  H  A  F   1219

ATTGGCGATGTCAAAGGTACCACGGTTGGGGGGTGTCATCACATTACATCAAAATACCTA  11100
 I  G  D  V  K  G  T  T  V  G  G  C  H  H  I  T  S  K  Y  L   1239

CCTAGGTCCCTGCCTAAGGACTCTGTTGCCGTAGTTGGAGTAAGTTCGCCCGGCAGGGCT  11160
 P  R  S  L  P  K  D  S  V  A  V  V  G  V  S  S  P  G  R  A   1259

GCTAAAGCCGTGTGCACTCTCACCGATGTGTACCTCCCCGAACTCCGGCCATATCTGCAA  11220
 A  K  A  V  C  T  L  T  D  V  Y  L  P  E  L  R  P  Y  L  Q   1279

CCTGAGACGGCATCAAAATGCTGGAAACTCAAATTAGACTTCAGGGACGTCCGACTAATG  11280
 P  E  T  A  S  K  C  W  K  L  K  L  D  F  R  D  V  R  L  M   1299
```

Fig. 1(13)

```
GTCTGGAAAGGAGCCACCGCCTATTTCCAGTTGGAAGGGCTTACATGGTCGGCGCTGCCC  11340
 V  W  K  G  A  T  A  Y  F  Q  L  E  G  L  T  W  S  A  L  P    1319

              C
GACTATGCCAGGTTTATTCAGCTGCCCAAGGATGCCGTTGTATACATTGATCCGTGTATA  11400
 D  Y  A  R  F  I  Q  L  P  K  D  A  V  V  Y  I  D  P  C  I    1339

GGACCGGCAACAGCCAACCGTAAGGTCGTGCGAACCACAGACTGGCGGGCCGACCTGGCA  11460
 G  P  A  T  A  N  R  K  V  V  R  T  T  D  W  R  A  D  L  A    1359

GTGACACCGTATGATTACGGTGCCCAGAACATTTTGACAACAGCCTGGTTCGAGGACCTC  11520
 V  T  P  Y  D  Y  G  A  Q  N  I  L  T  T  A  W  F  E  D  L    1379

GGGCCGCAGTGGAAGATTTTGGGGTTGCAGCCCTTTAGGCGAGCATTTGGCTTTGAAAAC  11580
 G  P  Q  W  K  I  L  G  L  Q  P  F  R  R  A  F  G  F  E  N    1399

ACTGAGGATTGGGCAATCCTTGCACGCCGTATGAATGACGGCAAGGACTACACTGACTAT  11640
 T  E  D  W  A  I  L  A  R  R  M  N  D  G  K  D  Y  T  D  Y    1419

AACTGGAACTGTGTTCGAGAACGCCCACACGCCATCTACGGGCGTGCTCGTGACCATACG  11700
 N  W  N  C  V  R  E  R  P  H  A  I  Y  G  R  A  R  D  H  T    1439

TATCATTTTGCCCCTGGCACAGAATTGCAGGTAGAGCTAGGTAAACCCCGGCTGCCGCCT  11760
 Y  H  F  A  P  G  T  E  L  Q  V  E  L  G  K  P  R  L  P  P    1459

GGGCAAGTGCCGTGAATTCGGGGGTGATGCAATGGGGTCACTGTGGAGTAAAATCAGCCAG  11820
 G  Q  V  P  -                                                  1463
             ORF2          M  Q  W  G  H  C  G  V  K  S  A  S     12

                                                  T
CTGTTCGTGGACGCCTTCACTGAGTTCCTTGTTAGTGTGGTTGATATTGCCATTTTCCTT  11880
  C  S  W  T  P  S  L  S  S  L  L  V  W  L  I  L  P  F  S  L    32
                                                     S

GCCATACTGTTTGGGTTCACCGTCGCAGGATGGTTACTGGTCTTTCTTCTCAGAGTGGTT  11940
  P  Y  C  L  G  S  P  S  Q  D  G  Y  W  S  F  F  S  E  W  F    52

TGCTCCGCGCTTCTCCGTTCGCGCTCTGCCATTCACTCTCCCGAACTATCGAAGGTCCTA  12000
  A  P  R  F  S  V  R  A  L  P  F  T  L  P  N  Y  R  R  S  Y    72

TGAAGGCTTGTTGCCCAACTGCAGACCGGATGTCCCACAATTTGCAGTCAAGCACCCATT  12060
  E  G  L  L  P  N  C  R  P  D  V  P  Q  F  A  V  K  H  P  L    92

  C                                              G
GGGTATGTTTTGGCACATGCGAGTTTCCCACTTGATTGATGAGATGGTCTCTCGTCGCAT  12120
  G  M  F  W  H  M  R  V  S  H  L  I  D  E  M  V  S  R  R  I   112
                                                 V
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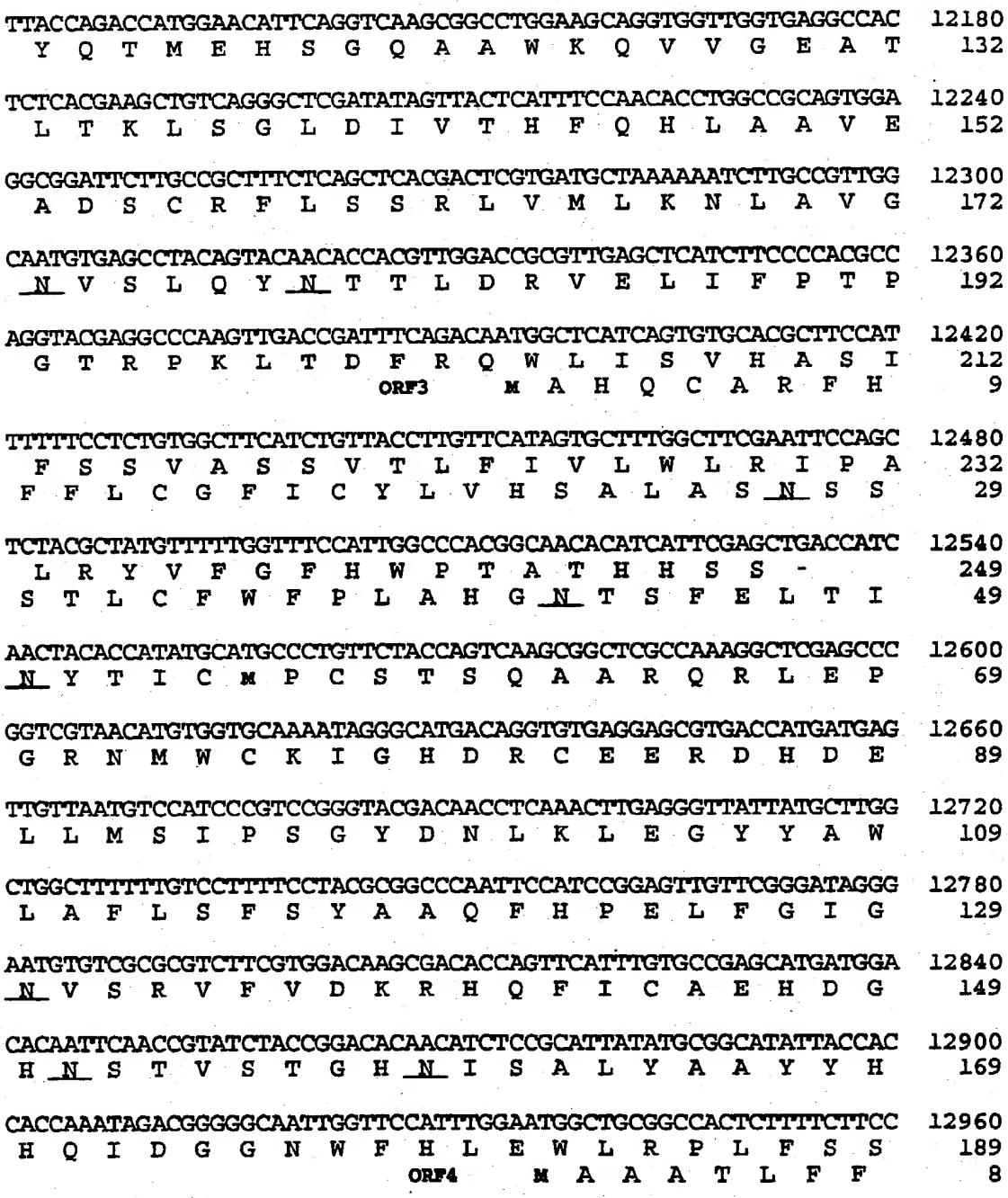
Fig. 1(14)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L T K L S G L D I V T H F Q H L A A V E 152
GGCGGATTCTTGCCGCTTTCTCAGCTCACGACTCGTGATGCTAAAAAATCTTGCCGTTGG 12300
A D S C R F L S S R L V M L K N L A V G 172
CAATGTGAGCCTACAGTACAACACCACGTTGGACCGCGTTGAGCTCATCTTCCCCACGCC 12360
N V S L Q Y N T T L D R V E L I F P T P 192
AGGTACGAGGCCCAAGTTGACCGATTTCAGACAATGGCTCATCAGTGTGCACGCTTCCAT 12420
G T R P K L T D F R Q W L I S V H A S I 212
ORF3 M A H Q C A R F H 9
TTTTTCCTCTGTGGCTTCATCTGTTACCTTGTTCATAGTGCTTTGGCTTCGAATTCCAGC 12480
F S S V A S S V T L F I V L W L R I P A 232
F F L C G F I C Y L V H S A L A S N S S 29
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L R Y V F G F H W P T A T H H S S - 249
S T L C F W F P L A H G N T S F E L T I 49
AACTACACCATATGCATGCCCTGTTCTACCAGTCAAGCGGCTCGCCAAAGGCTCGAGCCC 12600
N Y T I C M P C S T S Q A A R Q R L E P 69
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G R N M W C K I G H D R C E E R D H D E 89
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L L M S I P S G Y D N L K L E G Y Y A W 109
CTGGCTTTTTTGTCCTTTTCCTACGCGGCCCAATTCCATCCGGAGTTGTTCGGGATAGGG 12780
L A F L S F S Y A A Q F H P E L F G I G 129
AATGTGTCGCGCGTCTTCGTGGACAAGCGACACCAGTTCATTTGTGCCGAGCATGATGGA 12840
N V S R V F V D K R H Q F I C A E H D G 149
CACAATTCAACCGTATCTACCGGACACAACATCTCCGCATTATATGCGGCATATTACCAC 12900
H N S T V S T G H N I S A L Y A A Y Y H 169
CACCAAATAGACGGGGGCAATTGGTTCCATTTGGAATGGCTGCGGCCACTCTTTTCTTCC 12960
H Q I D G G N W F H L E W L R P L F S S 189
ORF4 M A A A T L F F 8

Fig. 1(15)

```
TGGCTGGTGCTCAACATATCATGGTTTCTGAGGCGTTCGCCTGTAAGCCCTGTTTCTCGA  13020
 W  L  V  L  N  I  S  W  F  L  R  R  S  P  V  S  P  V  S  R    209
L  A  G  A  Q  H  I  M  V  S  E  A  F  A  C  K  P  C  F  S     28

CGCATCTATCAGATATTGAGACCAACACGACCGCGGCTGCCGGTTTCATGGTCCTTCAGG  13080
 R  I  Y  Q  I  L  R  P  T  R  P  R  L  P  V  S  W  S  F  R    229
T  H  L  S  D  I  E  T  N  T  T  A  A  A  G  F  M  V  L  Q     48

ACATCAATTGTTTCCGACCTCACGGGGTCTCAGCAGCGCAAGAGAAAATTTCCTTCGGAA  13140
 T  S  I  V  S  D  L  T  G  S  Q  Q  R  K  R  K  F  P  S  E    249
D  I  N  C  F  R  P  H  G  V  S  A  A  Q  E  K  I  S  F  G     68

AGTCGTCCCAATGTCGTGAAGCCGTCGGTACTCCCCAGTACATCACGATAACGGCTAACG  13200
 S  R  P  N  V  V  K  P  S  V  L  P  S  T  S  R  -             265
K  S  S  Q  C  R  E  A  V  G  T  P  Q  Y  I  T  I  T  A  N     88

TGACCGACGAATCATACTTGTACAACGCGGACCTGCTGATGCTTTCTGCGTGCCTTTTCT  13260
V  T  D  E  S  Y  L  Y  N  A  D  L  L  M  L  S  A  C  L  F    108

ACGCCTCAGAAATGAGCGAGAAAGGCTTCAAAGTCATCTTTGGGAATGTCTCTGGCGTTG  13320
Y  A  S  E  M  S  E  K  G  F  K  V  I  F  G  N  V  S  G  V    128

TTTCTGCTTGTGTCAATTTCACAGATTATGTGGCCCATGTGACCCAACATACCCAGCAGC  13380
V  S  A  C  V  N  F  T  D  Y  V  A  H  V  T  Q  H  T  Q  Q    148

ATCATCTGGTAATTGATCACATTCGGTTGCTGCATTTCCTGACACCATCTGCAATGAGGT  13440
H  H  L  V  I  D  H  I  R  L  L  H  F  L  T  P  S  A  M  R    168

GGGCTACAACCATTGCTTGTTTGTTCGCCATTCTCTTGGCAATATGAGATGTTCTCACAA  13500
W  A  T  T  I  A  C  L  F  A  I  L  L  A  I  -                183
                                   ORF5      M  R  C  S  H  K     6

ATTGGGGCGTTTCTTGACTCCGCACTCTTGCTTCTGGTGGCTTTTTTTGCTGTGTACCGG  13560
  L  G  R  F  L  T  P  H  S  C  F  W  W  L  F  L  L  C  T  G    26

CTTGTCCTGGTCCTTTGCCGATGGCAACGGCGACAGCTCGACATACCAATACATATATAA  13620
  L  S  W  S  F  A  D  G  N  G  D  S  S  T  Y  Q  Y  I  Y  N    46

CTTGACGATATGCGAGCTGAATGGGACCGACTGGTTGTCCAGCCATTTTGGTTGGGCAGT  13680
  L  T  I  C  E  L  N  G  T  D  W  L  S  S  H  F  G  W  A  V    66

CGAGACCTTTGTGCTTTACCCGGTTGCCACTCATATCCTCTCACTGGGTTTTCTCACAAC  13740
  E  T  F  V  L  Y  P  V  A  T  H  I  L  S  L  G  F  L  T  T    86

AAGCCATTTTTTTGACGCGCTCGGTCTCGGCGCTGTATCCACTGCAGGATTTGTTGGCGG  13800
  S  H  F  F  D  A  L  G  L  G  A  V  S  T  A  G  F  V  G  G   106
```

Fig. 1(16)

```
GCGGTACGTACTCTGCAGCGTCTACGGCGCTTGTGCTTTCGCAGCGTTCGTATGTTTTGT  13860
  R  Y  V  L  C  S  V  Y  G  A  C  A  F  A  A  F  V  C  F  V    126

CATCCGTGCTGCTAAAAATTGCATGGCCTGCCGCTATGCCCGTACCCGGTTTACCAACTT  13920
  I  R  A  A  K  N  C  M  A  C  R  Y  A  R  T  R  F  T  N  F    146

CATTGTGGACGACCGGGGGAGAGTTCATCGATGGAAGTCTCCAATAGTGGTAGAAAAATT  13980
  I  V  D  D  R  G  R  V  H  R  W  K  S  P  I  V  V  E  K  L    166

GGGCAAAGCCGAAGTCGATGGCAACCTCGTCACCATCAAACATGTCGTCCTCGAAGGGGT  14040
  G  K  A  E  V  D  G  N  L  V  T  I  K  H  V  V  L  E  G  V    186

TAAAGCTCAACCCTTGACGAGGACTTCGGCTGAGCAATGGGAGGCCTAGACGATTTTTGC  14100
  K  A  Q  P  L  T  R  T  S  A  E  Q  W  E  A  -                201
                                ORF6     M  G  G  L  D  D  F  C    8

AACGATCCTATCGCCGCACAAAAGCTCGTGCTAGCCTTTAGCATCACATACACACCTATA  14160
  N  D  P  I  A  A  Q  K  L  V  L  A  F  S  I  T  Y  T  P  I    28

ATGATATACGCCCTTAAGGTGTCACGCGGCCGACTCCTGGGGCTGTTGCACATCCTAATA  14220
  M  I  Y  A  L  K  V  S  R  G  R  L  L  G  L  L  H  I  L  I    48

TTTCTGAACTGTTCCTTTACATTCGGATACATGACATATGTGCATTTTCAATCCACCAAC  14280
  F  L  N  C  S  F  T  F  G  Y  M  T  Y  V  H  F  Q  S  T  N    68

CGTGTCGCACTTACCCTGGGGGCTGTTGTCGCCCTTCTGTGGGGTGTTTACAGCTTCACA  14340
  R  V  A  L  T  L  G  A  V  V  A  L  L  W  G  V  Y  S  F  T    88

GAGTCATGGAAGTTTATCACTTCCAGATGCAGATTGTGTTGCCTTGGCCGGCGATACATT  14400
  E  S  W  K  F  I  T  S  R  C  R  L  C  C  L  G  R  R  Y  I   108

CTGGCCCCTGCCCATCACGTAGAAAGTGCTGCAGGTCTCCATTCAATCTCAGCGTCTGGT  14460
  L  A  P  A  H  H  V  E  S  A  A  G  L  H  S  I  S  A  S  G   128

AACCGAGCATACGCTGTGAGAAAGCCCGGACTAACATCAGTGAACGGCACTCTAGTACCA  14520
  N  R  A  Y  A  V  R  K  P  G  L  T  S  V  N  G  T  L  V  P   148

GGACTTCGGAGCCTCGTGCTGGGCGGCAAACGAGCTGTTAAACGAGGAGTGGTTAACCTC  14580
  G  L  R  S  L  V  L  G  G  K  R  A  V  K  R  G  V  V  N  L   168

GTCAAGTATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAAAGTACAGCTCCGATGGG  14640
  V  K  Y  G  R  -                                             173
 ORF7   M  A  G  K  N  Q  S  Q  K  K  K  K  S  T  A  P  M  G    18

GAATGGCCAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGATAAAGTCCCAGCG  14700
  N  G  Q  P  V  N  Q  L  C  Q  L  L  G  A  M  I  K  S  Q  R    38
```

Fig. 1(17)

```
                                T
CCAGCAACCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCCT  14760
  Q  Q  P  R  G  G  Q  A  K  K  K  K  P  E  K  P  H  F  P  L     58

GGCTGCTGAAGATGACATCCGGCACCACCTCACCCAGACTGAACGCTCCCTCTGCTTGCA  14820
  A  A  E  D  D  I  R  H  H  L  T  Q  T  E  R  S  L  C  L  Q     78

                                                  A
ATCGATCCAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAA  14880
  S  I  Q  T  A  F  N  Q  G  A  G  T  A  S  L  S  S  S  G  K     98

GGTCAGTTTTCAGGTTGAGTTTATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGT  14940
  V  S  F  Q  V  E  F  M  L  P  V  A  H  T  V  R  L  I  R  V    118

GACTTCTACATCCGCCAGTCAGGGTGCAAGTTAATTTGACAGTCAGGTGAATGGCCGCGA  15000
  T  S  T  S  A  S  Q  G  A  S  -                               128

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TTCAGGCAGGAACCATGTGACCGAAATTAAAAAAAAAAAAAAAAAAA               15088
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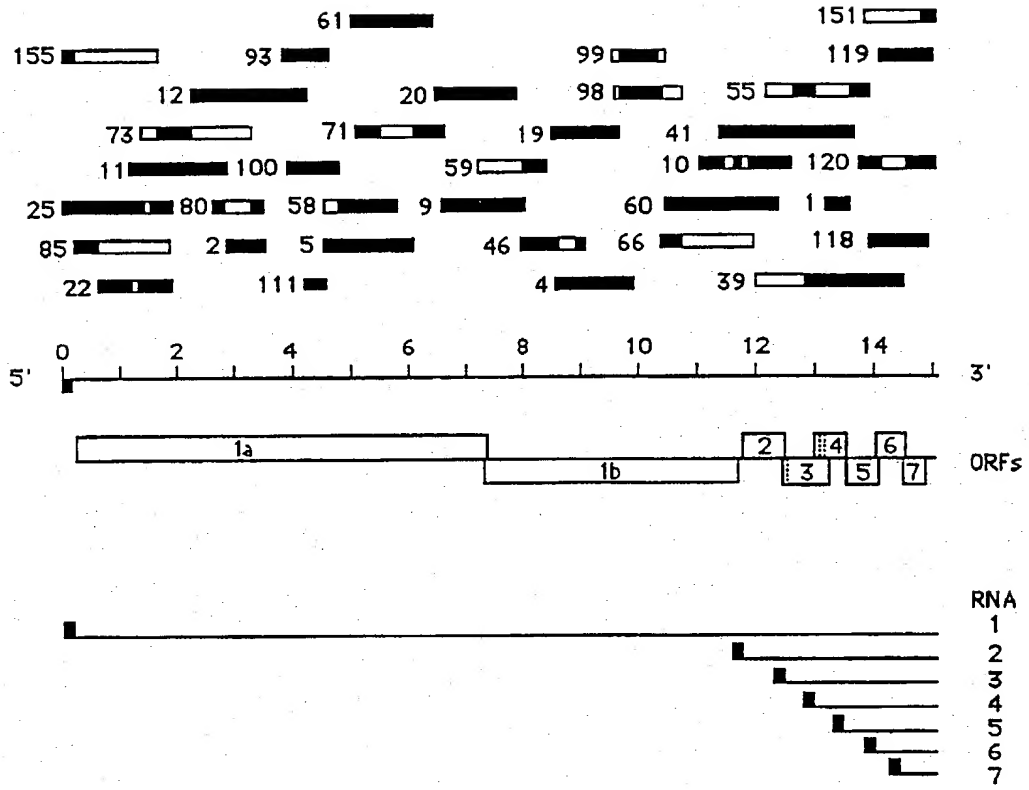
Fig. 2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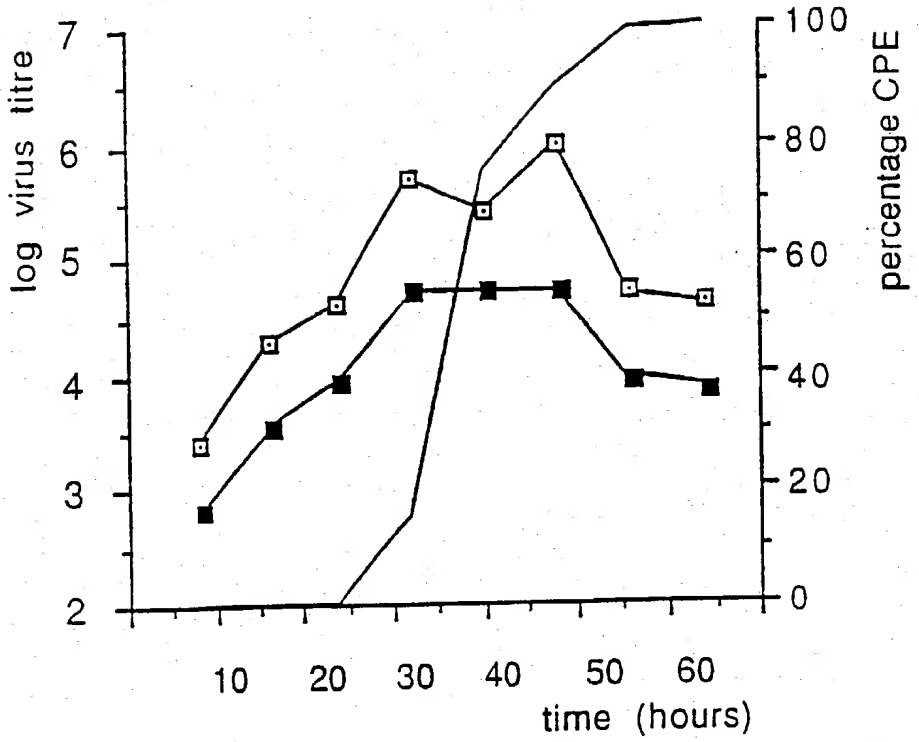
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6
5
4
3
2
100
80
60
40
20
0
percentage CPE
10
20
30
40
50
60
time (hours)

Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 92/00096

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/12; G01N33/569; C12N7/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; G01N ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X,P	THE VETERINARY RECORD Vol.128, no.24, 15 June 1991, LONDON, page 574; WENSVOORT G. ET AL. : " "Blue ear" disease of pigs." * column 1 *	1-26
X,P	THE VETERINARY QUARTERLY Vol.13, no.3, July 1991, pages 121-130; WENSVOORT G. ET AL.: " Mystery swine disease in the Netherlands: the isolation of Lelystad virus." * the whole document *	1-26

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 AUGUST 1992	15. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	AVEDIKIAN P.F.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	THE VETERINARY QUARTERLY Vol.13, no.3, July 1991, pages 131-136; TERPSTRA C. ET AL. : " Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled." * the whole document *	1-26
X,P	THE VETERINARY QUARTERLY Vol.13, no.3, July 1991, pages 137-143; POL J.M.A. ET AL.: " Pathological ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidemic abortion and respiratory syndrome (PEARS)) ." * the whole document *	1-26